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South Carolina State College (SCSC), in collaboration with the South Carolina Wildlife and Marine Resources Division (SCWMRD), initiated an investigation of the biosensing parameters regulating bacterial and larval attachment on submerged substrata. This program was designed to address a number of critical needs at both the state and national levels. These included: (1) addressing one or more of the current and continuing basic research needs of the Office of Naval Research (ONR) and (2) increasing the alarmingly small pool of minority students interested in pursuing careers in marine sciences through a coordinated research training program in marine science.

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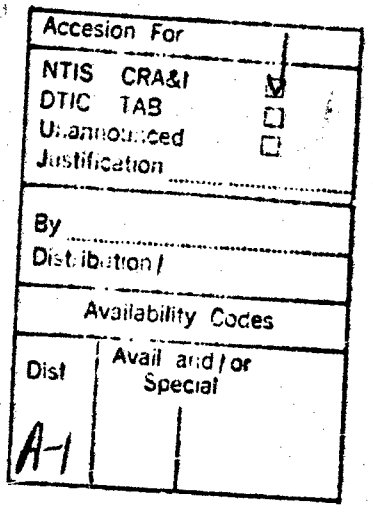


TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
RESEARCH OBJECTIVES	1
STUDENT TRAINING OBJECTIVES.	3
SUMMARY OF STUDENT TRAINING	5
EDUCATIONAL ACTIVITIES	10
SUMMARY OF RESEARCH ACCOMPLISHMENTS	12
ATTACHMENT OF MARINE BACTERIA TO POLYSTYRENE SURFACES	12
INTRODUCTION	12
MATERIALS AND METHODS	13
RESULTS	15
DISCUSSION	20
LITERATURE CITED	23
BACTERIAL EXTRUDANT PURIFICATION AND CHARACTERIZATION	24
SUMMARY	24
BACKGROUND/OBJECTIVES	24
METHODS	26
RESULTS	28
LITERATURE CITED	43

PAGE

CHARACTERIZATION AND IDENTIFICATION OF MARINE BACTERIA ASSOCIATED WITH BARNACLES IN CHARLESTON HARBOR, SOUTH CAROLINA 45

INTRODUCTION 45

MATERIALS AND METHODS 46

RESULTS 50

DISCUSSION 53

CONCLUSION 54

LITERATURE CITED 56

LARVAL ATTACHMENT BIOASSAYS 58

INTRODUCTION 58

SUMMARY OF LARVAL BIOASSAY RESEARCH 60

LITERATURE CITED 64

APPENDIX I 66

COMPARATIVE LARVAL SETTLEMENT BEHAVIOR IN THREE SPECIES OF BARNACLE (*BALANUS AMPHITRITE*,*B. EBURNEUS*, AND *B. IMPORVISUS*)

APPENDIX II 84

EFFECTS OF SURFACE-ASSOCIATED BACTERIA ATTACHMENT OF BARNACLE LARVAE

PAGE

APPENDIX III 93

PRESENTATIONS AND PUBLICATIONS (Last 12 Months)

APPENDIX IV 96

STATISTICAL ANALYSES FOR BACTERIAL ATTACHMENT STUDIES

INTRODUCTION

South Carolina State College (SCSC), in collaboration with the South Carolina Wildlife and Marine Resources Division (SCWMRD) initiated an investigation of the biosensing parameters regulating bacterial and larval attachment on submerged substrata. Further, this program was designed to address a number of critical needs at both the state and national levels. These included: (1) addressing one or more of the current and continuing basic research needs of the Office of Naval Research (ONR) and (2) increasing the alarmingly small pool of minority students interested in pursuing careers in marine sciences through a coordinated research training program in marine science.

The long-term objectives of this program focused on understanding and controlling the molecular processes which govern the sequence of interactions among substrata, bacteria, and invertebrate larvae in marine environments (biosensing). Control of these processes has application in marine environments where fouling organisms are a problem and the attachment or detachment of these organisms has economically important implications.

RESEARCH OBJECTIVES

The research program focused on controlling the settlement, attachment and detachment of marine bacteria and invertebrate larvae to substrata surfaces in a marine environment. We postulated a model in which molecular signals initiate a chain of biochemical and physiological processes within the microorganisms that mediate settling and attachment interactions among substrata, bacteria and invertebrate

larvae. Control of the settling and attachment processes may be achieved by sufficient understanding of the mechanisms to interrupt them at critical points. From this model, the following research objectives and hypotheses were formed for the first three years of this multi-year study:

1. Determine the parameters required for the preferential settlement of the marine bacteria *Pseudomonas fluorescens* (*P. fluorescens*) on polystyrene substrata. We hypothesized that the extrudants secreted by *P. fluorescens* and marine bacteria, to be identified (TBI) associated with *Balanus eburneus* (*B. eburneus*) serve as chemotaxic attractants which enhance the respective bacterial attachment to surfaces.

2. Determine the mechanisms of settling response and chemosensory recognition of cyprid larvae of *B. eburneus* to products of surface films of *P. fluorescens*.

Two research objectives for the first phase of the multiyear program included: (1) identification of individual positive or negative settling responses of cyprid larvae of *B. eburneus* to chemical extrudants from the local bacterium *P. fluorescens* and (2) determination of the influence of at least two bacterial extracts on the attachment of *B. eburneus* larvae. Our first null hypothesis was that the settling behavior of cyprid larvae of *B. eburneus* does not differ in response to chemical products of the bacterium *P. fluorescens*. Our second null hypothesis, tested during the first phase, was that attachment rates of cyprid larvae of *B. eburneus* do not vary in the presence of molecules isolated in the proposed studies of *P. fluorescens*.

STUDENT TRAINING OBJECTIVES

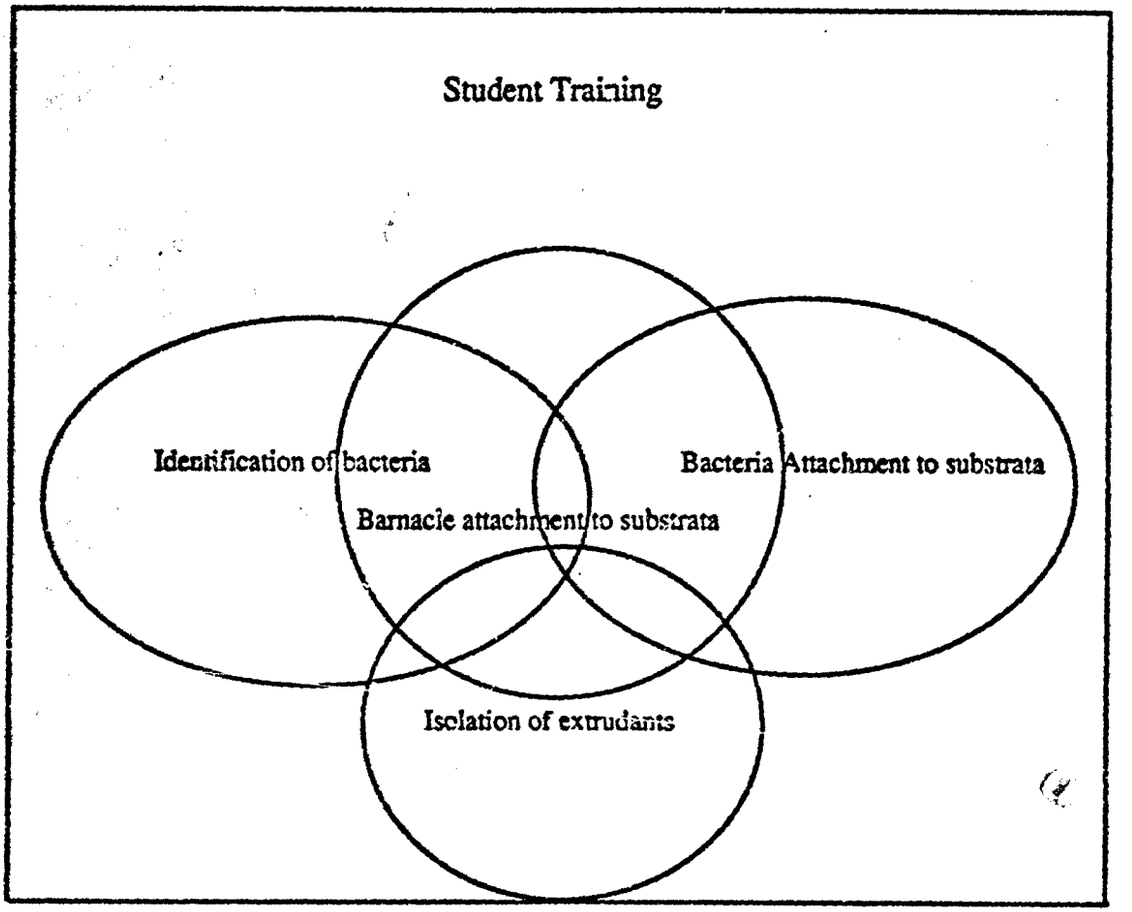
The aim of the collaborative relationship between the two institutions was to increase the number of minority students interested in pursuing careers in marine science. The program was based on combining the resources of the two state institutions. South Carolina State College would provide the educational facilities and SCWMRD would provide the initial research facilities. Research and teaching would be conducted at both facilities, where students would receive training (Diagram 1). The three primary objectives of the training program included:

1. To select science majors for research positions. Students would participate in all phases of the research, and, as a consequence, receive salaries during the summer and academic year, credit for their training, and guided instruction from SCSC and SCWMRD professionals. The students would present their findings at professional meetings such as those sponsored by the Southeastern Estuarine Research Society and the South Carolina Academy of Sciences.

2. To incorporate as many students (science majors) as logistically possible into a stimulating field application of marine science. This would be accomplished by utilizing the 52 ft. R/V Anita as a teaching platform for short cruises in Charleston Harbor. In addition, a marine science career day would be held to expose students to careers in marine science.

3. To have appropriate SCWMRD scientists teach courses in topics related to marine science for academic credit at SCSC.

Diagram 1. Integration Analysis of SCSU-SCWMRD Research Disciplines



Students received training in various aspects of the interrelated research as depicted above.

SUMMARY OF STUDENT TRAINING

Students were selected from a pool of interested science majors at SCSC and Claflin College. Selection criteria included grade point average, faculty recommendations, and interest in pursuing a career in marine science. Ms. Yvonne Bobo and Ms. Donnia Richardson of Marine Resource Research Institute (MRRI), as well as research supervisors at SCSU were instrumental in monitoring students' progress. They met with the students to discuss progress and problems, and coordinated evaluations of the students by the supervisors and evaluation of the program by the students. To date, eighteen students have participated in the program. Information on student participation and current status can be found in Table 1.

During the summer of 1990, a summer marine science experience was established at the S.C. Wildlife and Marine Resources Department (SCWMRD) and S.C. State College (SCSC). Initially, three students were selected to participate. While at SCWMRD, the students received an introduction to the facilities, staff and research being conducted at the Marine Resources Center in Charleston. Tours of the Marine Resources Research Institute (MRRI), the College of Charleston's Grice Marine Biological Laboratory, and the National Marine Fisheries Service. Each laboratory provided descriptions of the diversity of applied and academic research activities.

During a day-long cruise in the Charleston Harbor estuary, students participated in discussions of the effects of human development of coastal environments and the concomitant destruction of natural ecological systems. Students then rotated within various MRRI laboratories, where emphasis was placed on hands-on activities, i.e., seabird census, pathology and histology of samples of clams, oysters, and

Table 1. Student Participation in the ONR Program June 1990-May 1993

Information on Past and Present ONR Students

Name of Student	Time in ONR Program	Current Status
Melissa Sargent	June 90-May 91 (12 mo)	B.S. in Biology, SCSC Working at Roche Pharmaceuticals in NC
Arve Hammock	June 90-Dec 90 (7 mo)	B.S. in Chemistry, SCSC Working at Vintage Pharmaceuticals in NC
Brenda Frazier	June 90-May 91 (12 mo)	B.S. in Biology; worked for Dr. John Williams, Ecology, at SCSU for 14 months; currently working in Texas
Alexis Ware	June 91-July 92 (14 mo)	B.S. in Biology, SCSU Spring 93 [illegible] Duke Univ. Mar. Lab.; currently enrolled in the M.S. program in Wildlife and Fisheries at Texas [illegible]
Shauna Patterson	June 91-July 92 (14 mo)	Biology major at SCSU; working for Dr. Williams
Dionne Sumpter	June 91-Nov 91 (6 mo)	Biology major at SCSC
Tammie Terrell	June 91-Oct 91 (5 mo)	Biology major at SCSC
Chenise Lambert	June 91-July 91 (2 mo)	Biology major at SCSC
Dawn Evans	July 91-Oct 91 (4 mo)	B.S. in Biology, SCSU; working at Ethyl Chemical Plant, Orangeburg SC
Natombi Smith	June 91-May 92 (12 mo) Marine Biology	B.S. in Biology; Accepted at Xavier of Ohio in the M.S. program in
Leyana Lloyd	June 91-Jan 93 (20 mo)	Chemistry major at SCSU
George McCowan	June 92-Nov 92 (8 mo)	Biology major at SCSU; Spring 93 internship at MRRI
Michelle Haynes	June 92-May 93	Biology major at SCSU
Dhayalini Chandrasegaram	June 92-May 93	Biology/Chemistry major at Claflin College
Valinda Olds	April 93-May 93	Biology Ed. major at Claflin
Nalani Kalawe	April 93-May 93	Psychology major at SCSU
Scottie McDuffie	April 93-May 93	Biology major at SCSU
Aliyah Spruill	April 93-May 93	Biology major at SCSU

fish, clam and finfish mariculture, benthic ecology, estuarine communities, and sediment contaminants.

While at S.C. State College, the students learned a number of research techniques and methods which included growth and maintenance of bacteria, statistics, purification of the bacterial extrudants, and techniques of chromatography, microscopy and spectrophotometry. The summer experience stimulated the students to develop their research and critical thinking skills and to gain an appreciation of the various environmental factors which influence marine ecological systems.

During the academic year of 1990-91 the students continued their research training. In addition, during the spring semester of 1991, a course entitled, "Concepts in Marine Science", reintroduced as a team taught course at SCSC, involved all of the scientists in the project. The course was very successful in exposing students to various concepts in Marine Science, and most importantly, in recruiting students to participate in the ONR research/training program. Three of the five students who were enrolled in the course were selected to participate in the ONR program that began on June 10, 1991.

During the summer of 1991, seven S.C. State University (SCSU) students participated in a 6 week program at the SC Marine Resources Division (SCMRD) in Charleston, the Waddell Maricuiture Center in Bluffton,the Bear Island Game Management Preserve, and SC State College. Hands-on activities included: a cruise in Charleston Harbor, a survey of techniques for sampling and identifying marine organisms, an introduction to histological techniques for marine invertebrates, participation in aquaculture research, analysis of heavy metals and toxic organics in sediments and fish, and an introduction to bacteriological techniques. In addition, one student spent a week conducting barnacle research at SCMRD.

In addition, Donnia Richardson, a recent African-American college graduate was trained as a laboratory technician for the project at the SCWMRD laboratory. She participated in all aspects of the barnacle settlement research, field sampling, bacterial and larval culturing, and data analysis. In addition, a high school student assisted a College of Charleston graduate student in field studies of larval barnacle settlement.

During the academic year of 1991-1992 at S.C. State, the students learned a number of research techniques. These techniques included growth and maintenance of bacteria, measurement of bacterial attachment to polystyrene surfaces, purification of the bacterial extrudants, and techniques in chromatography, microscopy and spectrophotometry. A course entitled, "Concepts in Marine Science" was taught by Dr. Nancy O'Connor. The course enrolled 33 undergraduate students at SCSU. In addition, staff from Claflin College and SCMRD presented seminars on marine biology topics, and a visiting scientist program involving MRD staff was initiated at SCSU.

During the summer of 1992, one student from Claflin College and three students from SCSU were engaged in 7 weeks of rigorous research training at the ONR SCSU-Marine Microbiology Laboratory (ONR-SCSU MML). The students began their experience with an orientation to the laboratory. They received guidance and training in pertinent laboratory techniques. The intense, varied, and productive laboratory experiences included performing standard microbiological procedures and tests to separate and identify marine bacteria associated with barnacles attached to oyster shells in Charleston Harbor, participating in a journal club, collecting bacterial samples, visits to marine biology laboratories, conducting literature searches, writing weekly progress reports, training for poster and oral presentations and, performing bacterial attachment assays and extrudant isolation.

These students also spent one week at SCWMRD in Charleston for an intensive "hands-on" introduction to marine science research. Activities included a cruise in Charleston Harbor, sampling and identifying marine invertebrates and fishes, histological and life-history analyses of marine organisms, analyses of heavy metals and toxic organics in sediments and fish, and participation in aquaculture research. The students also performed a bioassay testing the effects of marine bacteria on larval barnacle attachment. Two students interested in careers in aquaculture worked full time on research projects at the SCWMRD's Waddell Mariculture Center. They monitored temperature and oxygen levels in experimental ponds stocked with marine shrimp, red drum or hybrid striped bass. They also harvested fish and shrimp in ponds and conducted feeding studies of Penaeid shrimp. In addition, they spent time at a privately owned shrimp farm where they observed the transfer of technology from the research environment to an actual commercial operation.

During the academic year, 1992-93, an introductory oceanography course (Concepts in Marine Science) was taught by SCWMRD staff at SCSU during the spring semester of 1993. Seminars on marine and environmental science topics featuring speakers from SCWMRD, University of S.C. , NMFS, and USFWS were presented to students and faculty at SCSU monthly. Also, students conducted research individually and presented their data at scientific meetings (Appendix III). Additionally, four new students were trained to assist in the identification of bacteria during the spring.

EDUCATIONAL ACTIVITIES

The educational component of the training program also was very important. It included an enrichment of the academic program at SCSU to increase student interest in marine science and interest in the ONR program. Marine Resources Research Institute (MRRI) staff taught three courses in introductory marine science and marine biology on the SCSU campus. A total of 47 students were enrolled. The courses included lectures, laboratories, field trips, and exercises in scientific communication. Additional educaticnal activities on the SCSU campus included lectures by MRRI staff in chemistry, ecology, and zoology courses on marine-related topics, and seminars about research activities at MRRI and other marine research institutions. Seminar speakers from a wide range of fields were enthusiastically received by students at SCSU and Claflin. The on-campus seminar series greatly increased the level of awareness of students and faculty of opportunities in marine and environmental science.

Communications were extremely important to the success of the program, in terms of student recruitment, dissemination of research results, and interactions with other institutions. Communications took many forms from the more formal, regular monthly meetings to critique progress in research and student training and planning of future

activities, to informal exchanges to keep students and the public informed of program activities. A program newsletter, "Synergy", with a current distribution list of 150, was sent to interested persons, institutions, and agencies at least three times a year. Newsletter emphasis was usually on student progress, potential opportunities for students and staff, as well as research progress. In addition, an award-winning video describing the program's student training activities was produced by the Conservation, Education and Communications section of the SCWMRD, and an article describing program activities appeared in the spring 1993 edition of the newspaper of the SCWMRD.

SUMMARY OF RESEARCH ACCOMPLISHMENTS

ATTACHMENT OF MARINE BACTERIA TO POLYSTYRENE SURFACES

INTRODUCTION

The attachment of bacteria to various submerged substrata has been of interest because of its importance in the process of biofouling. Sieburth (1979) reported that marine bacteria are the first microorganisms to colonize new aquatic surfaces whether animate or inanimate. Furthermore, attachment is preceded by the rapid adsorption of a thin organic film composed of polysaccharide-protein complexes which are released into the ocean as a result of phytoplankton metabolism and decay (Sieburth 1979). Bacteria, however, show a greater selectivity toward animate surfaces such as oysters and marine microalgae (Paerl 1980, Tosteson et al. 1984, Morse 1985, and Weiner et al. 1989).

The attachment of bacteria to surfaces depends on a number of factors, including the properties of the substratum, the liquid phase, and the bacteria themselves (Pringle and Fletcher 1986) They also indicated that attachment was influenced by factors such as

surface charge, surface free energy and the presence of adsorbed macromolecules. Whitfield (1988) reported that initial bacterial cell attachment to marine substrata is reversible and is often followed by permanent attachment. Tosteson (1984) revealed that some bacterial species exhibited a preference for attachment to specific substrata which had been exposed to extracellular polysaccharides (EPS). However, Fletcher and Pringle (1985) reported that bacteria exhibit selective attachment to substrata even in the absence of EPS. Therefore, the goals of this investigation were to compare the attachment properties of marine bacteria to polystyrene surfaces at specific time intervals.

MATERIALS AND METHODS

I. Growth Conditions

Studies were conducted to determine the attachment properties of *Pseudomonas fluorescens*, *Deleya marina*, and *Alteromonas macleodi* on polystyrene surfaces. Two flasks containing 250 ml of Zobell's Marine Broth (ZMB) were inoculated with 2.5 ml of *Deleya marina* and *Alteromonas macleodi* and one flask containing Costerton's medium was inoculated with 2.5 ml of *P. fluorescens*. The cells were grown for 24 and 48 hr. periods in an orbital water bath at 25 C. The cells

in each flask were then poured into three 250 ml sterile centrifuge tubes, and centrifuged for 10 min. at 10,000 rpm to pellet the cells. The supernatant was decanted and the cell pellet was resuspended in 100 ml of sterile sea water. The experiment was conducted in triplicate.

II. Attachment Studies

A. In the Absence of Supernatant

Attachment studies were carried out according to a modification of the method of McEldowney and Fletcher (1988). Polystyrene cuvettes were labeled in triplicate for time periods of 0.25, 0.50, 1, 2, 24, 72, 96, and 120 hours, respectively. Next, 3 ml of each cell type were added to cuvettes and were allowed to incubate at 25 C, for the indicated time intervals. After incubation, each cuvette was rinsed three times with distilled water to remove loosely attached cells. The cuvettes were then fixed with Bouin's fixative for 30 minutes. The cuvettes containing the attached cells were stained with crystal violet for 2 min. After staining, the cuvettes were rinsed 6 times with distilled water. Absorbance readings of the cuvettes were taken at 590 nm.

B. In the Presence of Supernatant

The attachment studies were carried out as above except, the cuvettes were exposed respectively to supernatant from *Deleya marina*, and *Alteromonas macleodi* and allowed to adsorb prior to the addition of *Deleya marina*, and *Alteromonas macleodi* cells. In addition, tests were conducted with supernatant that was filtered in a 0.2 micrometer pore filter.

RESULTS

Results indicated that *A. macleodi* demonstrated the highest attachment properties of the three bacteria tested for both the 24 and 48 hr. cultures. The O.D. readings were as high as 0.88 within the first 2 hours. In contrast, *P. fluorescens* and *D. marina* had O.D. readings of 0.6 and 0.22 for 24 hr. cultures, respectively (Fig. 1). For 48 hr. cultures (Fig. 1), *P. fluorescens* and *D. marina* demonstrated readings of 0.38 and 0.35 respectively at the 2 hr. interval (Fig. 2). At 72 hr., *P. fluorescens* showed the highest readings of the three bacteria, 0.85 for the 48 hr culture and 0.6 for the 24 hr. grown culture. *A. macleodi* and *D. marina* showed

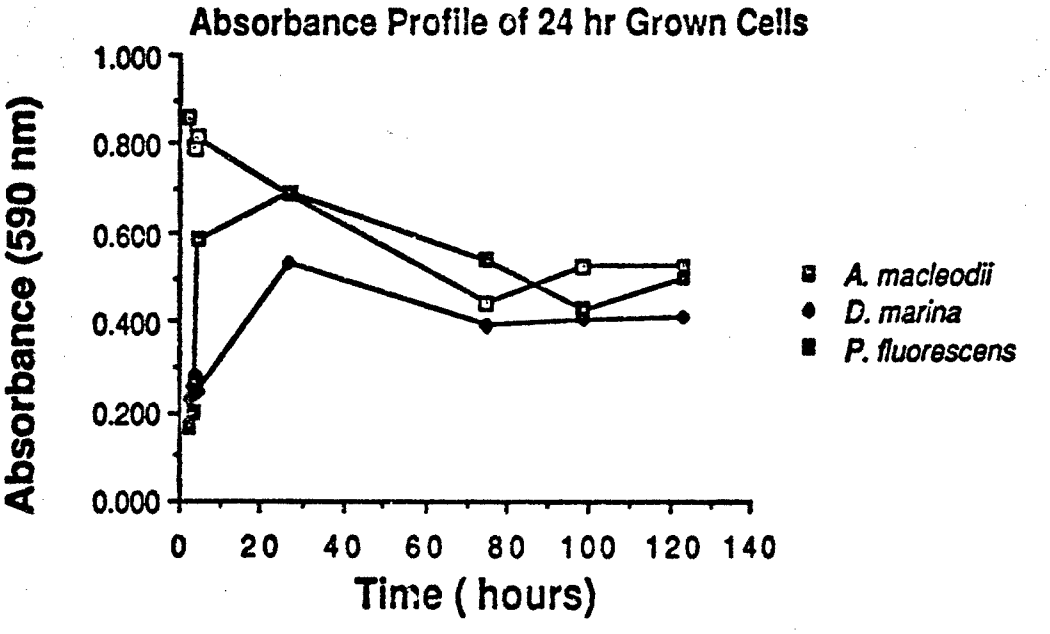


FIGURE 1

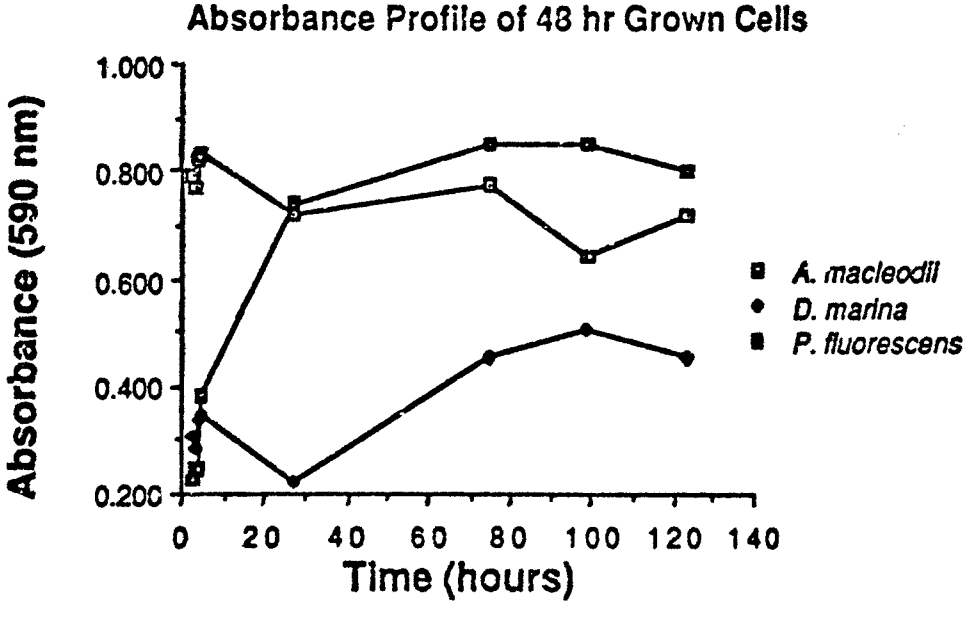


FIGURE 2

readings of 0.75 and 0.45 respectively, for 48 hr. grown cultures. Further, *A. macleodi* and *D. marina* had O.D. readings of 0.5 and 0.4, respectively, for 24 hr. grown cultures.

A. macleodii showed the highest attachment properties of the three bacteria, within the first two hours; in contrast, *P.fluorescens* cells reached their highest attachment at 24 hr. for the 24 hr. grown culture and 72 hr. for the 48 hr. grown culture. *D. marina* demonstrated the lowest attachment properties of the three bacteria.

Statistical analyses were performed on the three cultures for 24 hr. and 48 hr. cultures at the various time periods (Appendix III).

To determine the effects of bacterial supernatant on the attachment of bacteria to polystyrene surfaces, the cuvettes were exposed to the supernatant of *Deleya marina* and *Alteromonas macleodi* for 2 hours prior to adding the cells. *Deleya marina* and *Alteromonas macleodi* showed a decrease in attachment in the presence of supernatant, filtered and unfiltered (Figures 3 and 4).

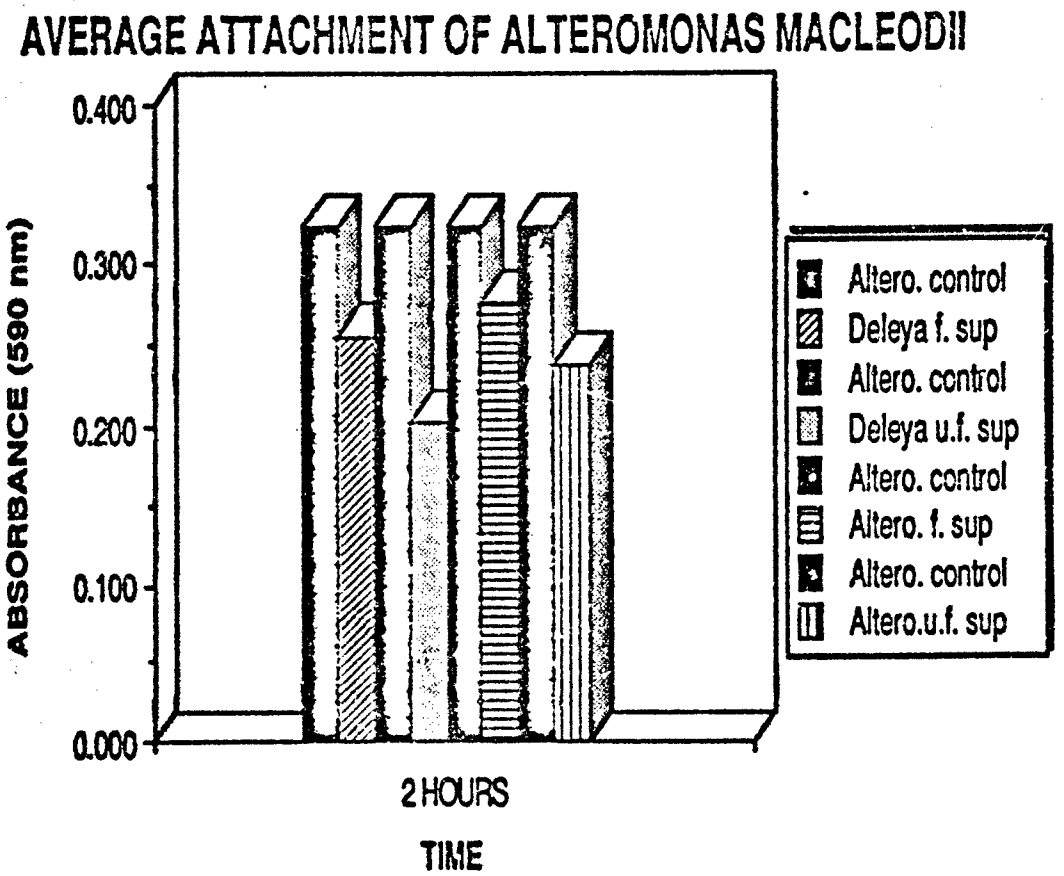


FIGURE 3

f. sup= filtered supernatant
u.f. sup= unfiltered supernatant

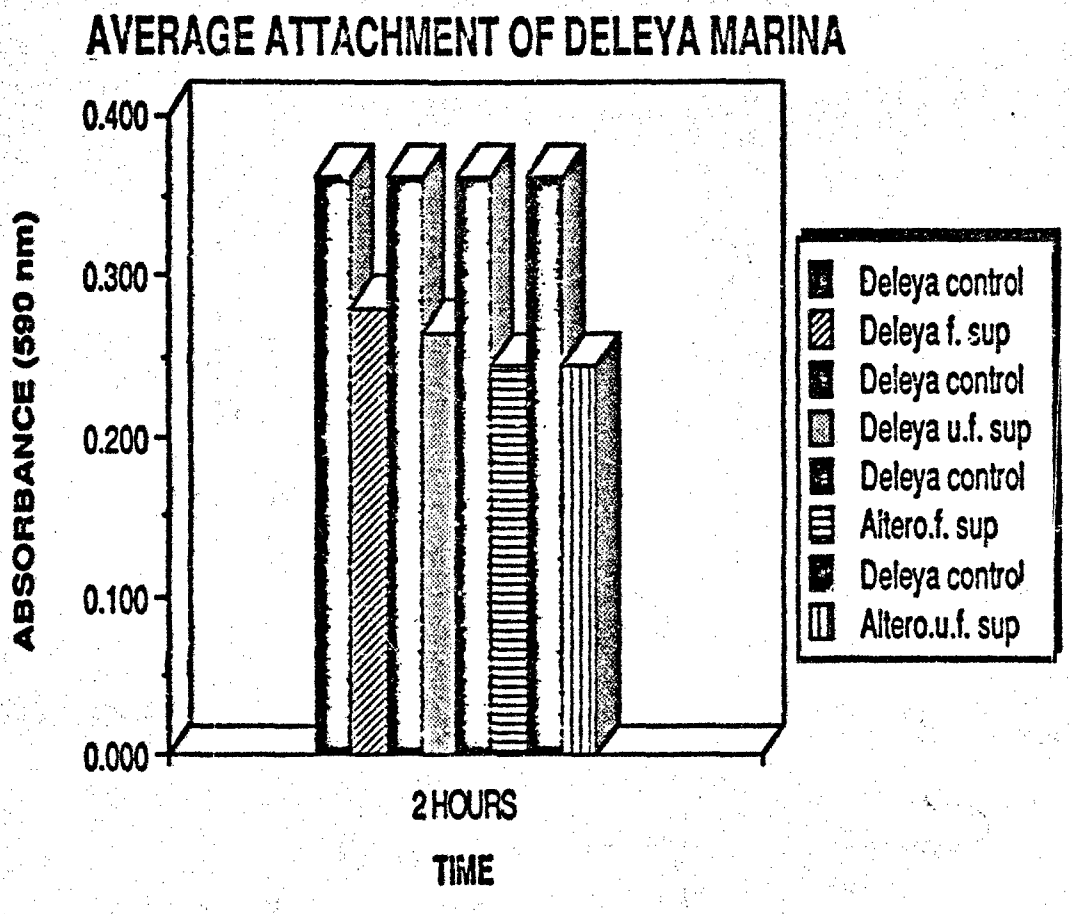


FIGURE 4

f. sup= filtered supernatant
u.f. sup= unfiltered supernatant

DISCUSSION

Attachment studies indicated that within 15 min. marine bacteria attach to polystyrene surfaces, although the degree in which they attach varies. Further, the attachment properties for theses organisms did not remain constant over the experimental time intervals. The null hypothesis for the experimental data is that the mean adsorbance for each bacterial species is equal for the various time periods at the 0.05 level. Results indicate that there is a significant difference between the means ($p = 0.047$) for *A. macleodii* grown for 24 hr. at the various time periods. In contrast, *A. macleodii*, grown for 48 hr., showed no significant difference between the means ($p = .995$). Similarly, *D. marina* grown for 24 hr. and 48 hr., and *P. fluorescence* grown for 24 hr., showed no significant difference between the means. However, *P. fluorescens* , grown for 48 hr., showed a significant difference at a p value of 0.00.

Comparisons were made of differences between the means of *A. macleodii*, *D. marina*, and *P. fluorescence* at the same time interval for cells grown for 24 and 48 hrs. The results indicated that there was a significant difference between the means of *A. macleodii*, *D. marina*, and *P. fluorescence* cells grown for 24 hr. at the following intervals: 0.25 hr., 0.5, 1, and 2 hr., respectively. In, contrast there was no significant

difference for the time intervals of 24, 72, 96, and 120 hr. There was a significant difference between the means of *A. macleodii*, *D. marina*, and *P. fluorescence* grown for 48 hr. at time intervals 0.25, 0.5, 1, 2, 24, and 48 hrs. respectively. However, for the time intervals 96 and 120 hr. there was not a significant difference between the means.

Results indicate that the supernatants of *Deleya marina* and *Alteromonas macleodi* decrease the attachment of these cells to polystyrene. We suspect that the supernatant may contain some components that binds to the polystyrene cuvette and interferes with the adsorption capacity of the cells. These experiments were conducted several times with reproducible results.

These studies were carried out in cuvettes in a vertical position, and the attachment of cells was measured on the sides of the cuvettes, gravitational forces were not considered to affect vertical cell adhesion. Moreover, our data suggests that surfaces do not have to be preadsorbed with an organic film in order for bacterial attachment to occur. Therefore, in order to inhibit the attachment of the bacteria to a surface, the surface must be treated before exposure to the marine environment. Although bacteria are minute, they play an important role in modifying

surfaces and thereby promote the biofouling of larger invertebrates on surfaces. Information derived from this study will be compiled and submitted to the Journal of Applied and Environmental Microbiology.

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BACTERIAL EXTRUDANT PURIFICATION & CHARACTERIZATION

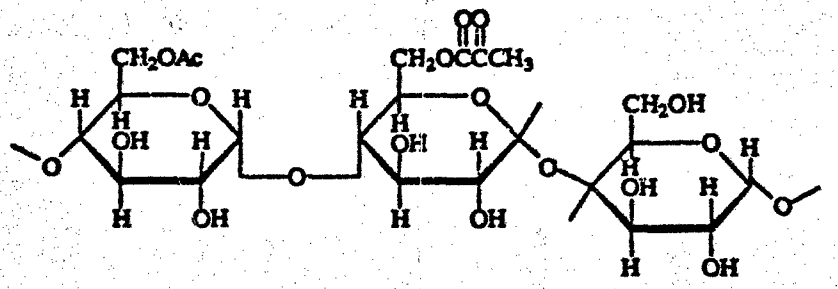
SUMMARY

Using a combination of dialysis, enzyme degradation, solvent precipitation and size-exclusion chromatography, supernatants of *Pseudomonas fluorescens* (PF), *Alteromonas macleodii* (AM), and three bacterial species isolated from Charleston harbor (OSM, OSL 2171, OSL 0031) have been enriched in high-molecular-weight extracellular polysaccharides (EPS). Based on colorimetric analyses of supernatants at various stages of purification, 44-90% of soluble carbohydrate and 25-27 % of soluble protein have molecular weights greater than 50 K. All supernatants contain some carbohydrate with molecular weights of at least two million. Growth curves for the latter four species, determined in Zobell's marine broth (ZMB) show significantly fewer bacteria and colloidal bacterial metabolites for AM and OSL 0031.

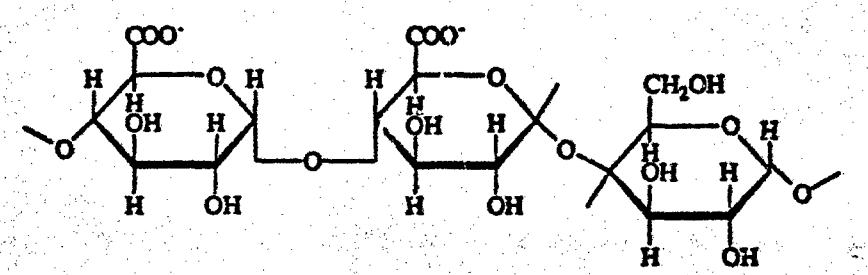
BACKGROUND/OBJECTIVES

Bacterial adhesion to marine surfaces is a function of surface properties, seawater composition, and bacterial physiology and species. Along with other metabolites, many bacteria extrude (EPS) which mediate their permanent attachment and comprise the structural matrix of a biofilm. The biofilm hosts and protects bacteria and other microbes. *Pseudomonas fluorescens*, a primary settler present in southern marine waters, extrudes EPS which after purification, hydrolyses producing D-glucose, D-galactose, acetate and pyruvate. Read and Costerton (1987) did not detect uronic acids. Thus *Pseudomonas fluorescens* EPS is a

heteropolymer (~ 2 million daltons) of D-glucose, and D-galactose esterified with acetic and pyruvic acids. In addition to bacterial adhesion, bacterial films and supernatants are reported to affect the settlement of oyster and barnacle larvae on polystyrene. Several species of barnacle larvae settle in larger numbers on polystyrene surfaces containing *Alteromonas macleodii* than on polystyrene controls.



Neutral Heteropolysaccharide



Anionic Polysaccharide

We sought to isolate and identify EPS from *Pseudomonas fluorescens* and other bacteria taken from Charleston harbor to determine the effect of preadsorbed EPS on the attachment of *Pseudomonas fluorescens* to polystyrene cuvette surfaces. Bacterial attachment was measured spectrophotometrically via the method of Pringle and Fletcher (1983). Purified and partially-purified were also be incorporated into experiments examining the settling of several barnacle larvae species on polystyrene and glass and how this settlement was affected by bacteria and bacterial biofilm components.

METHODS

Pseudomonas fluorescens 105 (PF), and *Alteromonas macleodii*, obtained from Presque Isle Cultures, were stored at -20 C. Periodically, a portion of this PF stock was cultured overnight in a medium described by Read and Costerton (1987), containing $NaHPO_4$ (10.0 g/L), KH_2PO_4 (3.0 g/L), K_2SO_4 (1.0 g/L), NaCl (1.0 g/L), $MgSO_4$ (0.20 g/L), $CaCl_2$ (0.10 g/L), $FeSO_4$ (0.01 g/L), casamino acids (3.0 g/L) and D-glucose (20.0 g/L). To 250 ml of this sterile solution, pH 7.0, were added 2.5 ml of *Pseudomonas fluorescens* overnight culture. The mixture was grown at 25 C for 24-96 hours with constant shaking. Three species of bacteria were taken from cyster shells in the mid-intertidal (OSM) and the low-intertidal region (OSL 0031 and OSL 2171) behind Grice Marine Biological Laboratory located adjacent to Charleston harbor. These three species and AM were cultured overnight. Five milliliters of the respective overnight culture were added to 250 ml of Zobell's marine ZMB and were grown at 25 C for 24-96 hours. The ZMB consisted of bacteriological peptone (5 g/L), yeast extract (1 g/L), ammonium nitrate (0.0016 g /L), boric acid (.022 g/L), calcium chloride (1.8 g/L), disodium hydrogen phosphate (0.008 g/L), ferric citrate (0.1 g/L), magnesium chloride (8.8 g/L), potassium bromide (.08 g/L), potassium chloride (0.55 g/L), sodium bicarbonate (0.16 g/L), sodium chloride (19.45 g/L), sodium fluoride (0.0024 g/L), liquid sodium silicate (0.004 g/L), sodium sulphate (0.324 g/L), strontium chloride (0.034 g/L). Growth curves were determined at 25 C by periodically withdrawing 3 ml aliquots of the growth supernatant and measuring the absorbance at 530 nm using ZMB as a blank.

We monitored the chemical composition of bacterial extrudants at various points during the purification using colorimetric assays for protein (Lowry et al. 1951), carbohydrate (DuBois et al. 1956), and uronic acids (Blumenkrantz and Asboe-Hansen 1972) and DNA (Burton 1955). These assays are sensitive at the µg level but generally are quantitative only on pure samples. Blumenkrantz and Asboe-Hansen's method for determination of uronic acids was insensitive to hexoses and pentoses. The phenol-sulfuric acid test for carbohydrate also responded to DNA but was insensitive to protein. Lowry's protein test was insensitive to both carbohydrate and DNA.

The general scheme for purification of *Pseudomonas fluorescens* and other bacterial species EPS is presented (Fig. 1). As a final purification step, samples were chromatographed on a 2.5 cm diameter, 60 cm length Sepharose 4B size exclusion column with an elutant of 0.05 M ammonium acetate buffer containing 0.02% sodium azide as a preservative. Molecules of greater than two million molecular weight elute were in the void volume (~95 ml) of the column while smaller molecules eluted with progressively larger volumes up to the total column volume of 300 ml. In some cases, fractions of interest were combined, dialyzed to remove salts, and further concentrated by either rotary evaporator or lyophilization to yield white or grayish-white solids. Solutions and solids were stored at -20 C to retard chemical or bacterial degradation of samples.

Separation of some samples was attempted by HPLC on a SynChropak GPC 500 size-exclusion column (250 x 4.6 mm dia.) eluting with a 0.1 M phosphate buffer (KH_2PO_4 , K_2HPO_4) pH 6.6 and with UV detection at 254 nm.

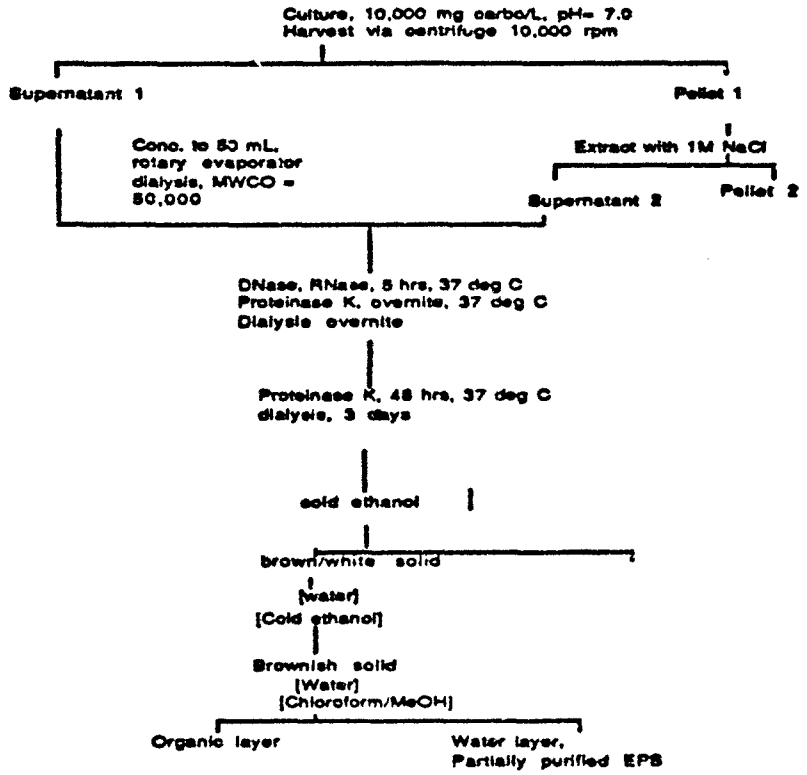


Figure 1. Flow diagram for EPS isolation and purification

RESULTS

Least-square calibration curves obtained with standards indicated below were linear with R^2 equal to unity (a value of one indicates no deviation of the experimental points from the least square line).

Standard	Wavelength	Least Square Equation	R^2
Total Carbohydrate			
1. glucose	490 nm	A = -0.021 + 0.006 (ug glucose)	1.00
Dextran mw = 2 million	490 nm	A = -0.01 + 0.01 (ug Dextran)	1.00
Total Protein			
2. bovine serum albumin	750 nm	A = 0.023 + 0.003 (ug protein)	1.00
Total uronic acids			
3. glucuronic acid	520 nm	A = -0.003 + 0.027 (ug uronic acid)	1.00
4. DNA 2-deoxy-D-ribose	595 nm	A = 0.009 + 0.005 (ug of DNA)	1.00

Horan (1986) reported that the phenol-sulfuric acid analysis of total carbohydrate affords three times the absorbance reading for glucose as for a typical bacterial EPS. As a check, we determined calibration curves for both glucose and dextran, a 2 million molecular-weight polysaccharide produced by *Leuconostoc mesenteroides* (Fig. 2). We found that glucose absorbance was 1.3 times that of dextran. The glucose standard therefore underestimates polymeric carbohydrate. Despite these findings, we have chosen, for convenience, to report total carbohydrate in mixtures based on the glucose standard. Additionally, in carbohydrate-nucleic acid mixtures the phenol-sulfuric acid assay responds to DNA; therefore, we regarded the test as indicating both carbohydrate and DNA. Most colorimetric analyses were done in triplicate and the averages reported.

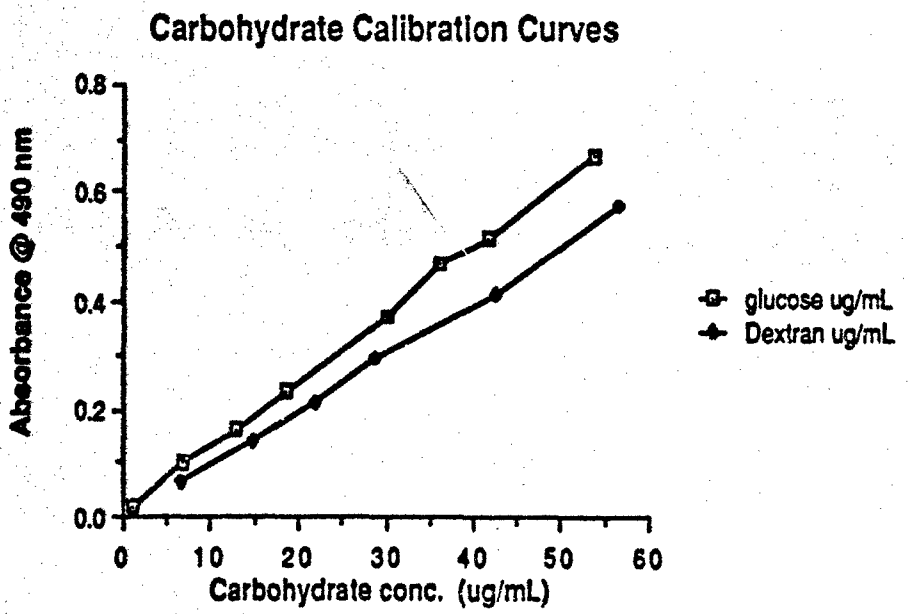


Figure 2. A Typical Colorimetric Calibration Curve

Most of carbohydrate and protein in bacterial ZMB supernatants were in solution. Washing the bacterial pellets of several species with 1 M

NaCl solution to remove tightly-held carbohydrate/protein afforded 1-4 % of supernatant carbohydrate and 0.5-1% of supernatant protein. Initially, partially-purified, yellow-brown EPS samples containing mg quantities of carbohydrate were stored at 12 C for periods of 2-3 months. Subsequent chromatographic work with these samples indicated no high-molecular-weight (HMW) EPS. This indicated some polysaccharide decomposition. To test EPS long-term stability, both carbohydrate and protein analyses were conducted.

	Carbohydrate		Protein	
Date	A @ 490 nm	mg	A @ 750 nm	mg
12-21-91	0.325 (0.025)	6.4 (0.46)	0.541 (0.003)	23 (0.11)
01-10-92	0.360 (0.011)	7.1 (0.21)	0.563 (0.002)	24 (0.09)
02-04-92	0.372 (0.005)	7.3 (0.08)	0.526 (0.008)	20 (0.31)

The standard deviation of triplicate measurements, in parentheses, indicate representative precisions. No significant loss of material occurred over the 44 day period. In order to optimize EPS production for bacteria grown in ZMB, we determined a series of growth curves given in Figure 3.

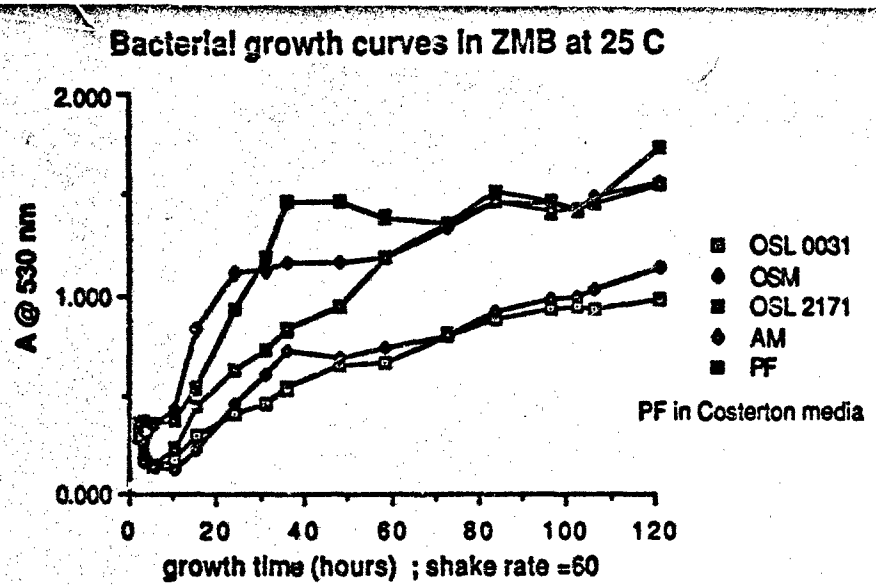


Figure 3. Bacterial growth curves in ZMB

Increasing absorbance at 530 nm represented increases in both bacteria concentration and colloidal (HMW) bacteria metabolites (Nelson et al. 1988) Early rapid absorbance increases were due primarily to bacterial growth, later increases reflected increasing concentrations of HMW carbohydrates, proteins, etc. In ZMB OSM, AM and OSL 0031 growth plateaued by 40 hours; OSL 2171 growth plateaued after 80 hours. After 100 hours of growth AM and OSL 0031 have significantly lower absorbances than the other bacterial species indicating less bacteria and/or polymeric bacterial metabolites. These two species (Table 1) also produced high ratios of protein/carbohydrate in the initial supernatant. One explanation for both observations is that OSL 0031 and AM initially produce relatively large amounts of small molecular-weight proteins which do not scatter light nor survive the dialysis purification step.

Table 1 summarizes the changes in carbohydrate and protein accompanying the various purification steps. The very large amounts of carbohydrate and protein in PF supernatants reflects the relatively large

TABLE 1. AMOUNTS OF CARBOHYDRATE/PROTEIN AT VARIOUS POINTS IN EPS PURIFICATION SCHEME

All species grown 100 hrs at 25 C in ZMB. AM grown for 24 hrs. PF grown in Costerton's growth medium.

Sample	OSM	OSL 0031	OSL 2171	PF	AM
Supernatant Concentrate					
Volume(ml)	54	53	25	53	-
Carbohydrate					
ug/ml	468	373	472	23,000	
total (mg)	23.9	19.8	11.8	1219	-
Protein					
ug/ml	1073	2833	3173	21,200	
total (mg)	54.7	150	79.3	1120	-
After dialysis (molecular wt. cutoff of 50 k)					
Carbohydrate					
ug/ml	370	175	184	216	
total(mg)	21.5	10.5	5.2	13.0	24.4
Protein					
ug/mL	235	688	773	187	
total(mg)	13.6	41.3	21.6	11.2	81.5
% HMW Carbo.	90	53	44	1.0	-
% HMW Protein	25	28	27	1.0	-
After Enzyme Degradation & Dialysis					
Carbohydrate					
total (mg)	15.7	8.6	3.0	8.2	-
Protein					
total (mg)	20.0	26.8	14.3	21.8	-
After Ethanol Precipitation & Chloroform extraction					
Carbohydrate					
total (mg)	11.2	4.06	2.94	6.35	4.83
Protein					
total (mg)	5.62	5.74	0.88	6.3	1.16

amounts of these monomeric substances in the original growth media. We regard levels of carbohydrate and protein after dialysis to reflect soluble bacterial metabolites since the literature reports these compounds to be of high molecular weight. We have analyzed the water (4-8 liters) in which the dialysis bags are suspended and always find very large amounts of both carbohydrate and protein. Ratios of carbohydrate in water: carbohydrate retained in dialysis bags are 15:1 for PF, 9:1 for OSL 2171 and 8:1 for OSM 071492, demonstrating that most of the carbohydrate had passed through the dialysis membrane. The enzyme degradation should significantly reduce DNA, RNA and protein and in the past we have seen protein reductions of 50-66%. With OSM and PF, protein increased by an amount which was approximately the mass of the proteinaceous enzymes added. With OSL 0031 and OSL 2171 the decrease in protein was 35 and 34%, respectively. We utilized two enzymatic reductions, DNase and RNase for nucleic acids and Proteinase K for protein degradation. In several successive nucleic acid degradations, following the procedure of Costerton, we obtained 6.4 (+0.5) mg and 6.7 (+0.4) mg of carbohydrate after dialysis. Since carbohydrate analysis also detects nucleic acids, the lack of any significant decrease in carbohydrate with successive degradations implies the enzymatic reaction has completely consumed polymeric nucleic acids with molecular weights greater than 50 K. The highly variable protein degradation with Proteinase K probably reflects the degradation solution components and their concentrations. Our initial protein degradation solutions contained EDTA and SDS detergent without added buffer no attempt was made to adjust the pH. Subsequently, the procedure of Spiro (1976) employing calcium acetate, and a Tris buffer of pH 7.8 was used. Optimum condition for the protein degradation are still being investigated.

The addition of cold ethanol to the supernatant preferentially precipitates HMW carbohydrate but if HMW protein were present it also would precipitate. Our chloroform/methanol extraction (Table1) appeared quite effective in removing protein. For comparison, Platt and Geesey (1985) have reported yields of carbohydrate and protein at several steps in a EPS purification.

Size exclusion chromatography on Sepharose 4B is the final step in EPS purification. The chromatogram of partially purified PF EPS is depicted in Figure 4.

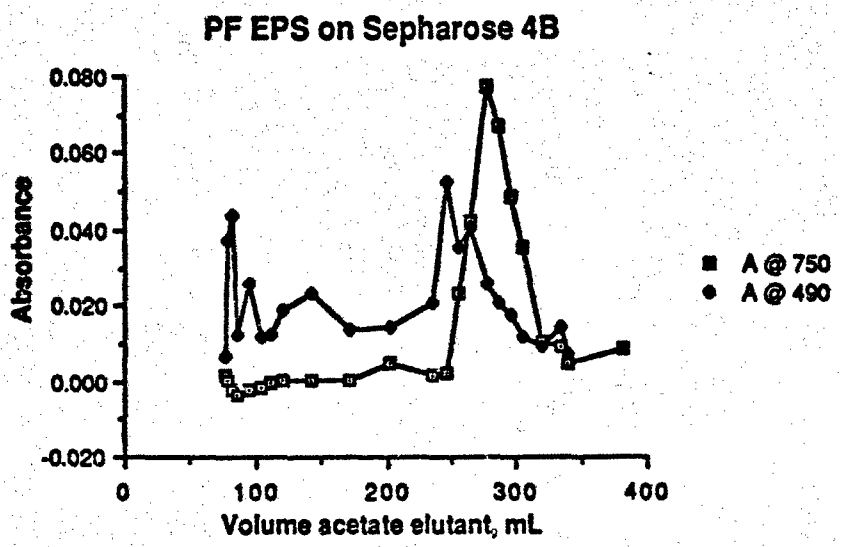
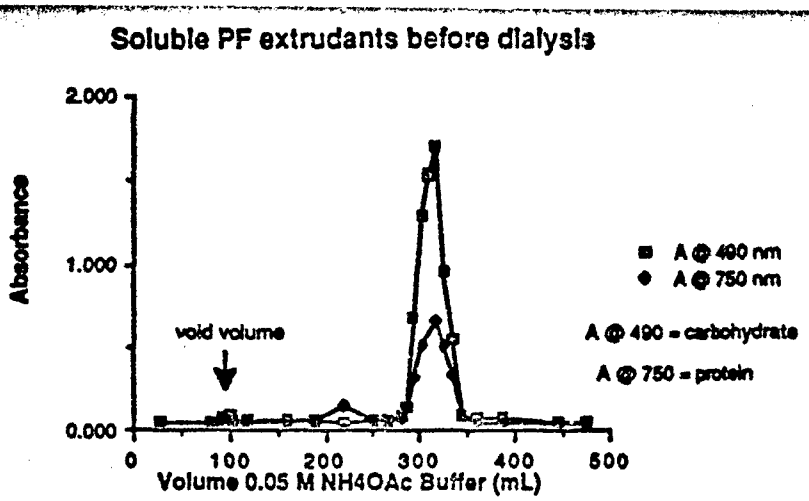
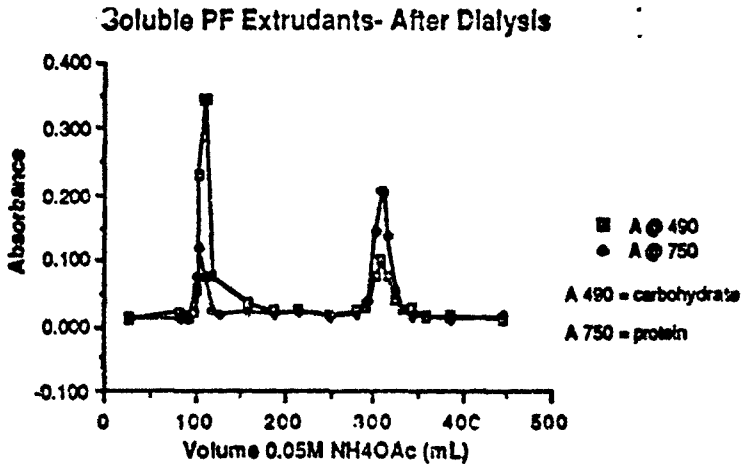


Figure 4. Chromatogram of partially purified PF (FW1353) on Sepharose 4B

Column void volume (89 ml) was determined by eluting Blue Dextran, molecular weight two million, and monitoring its concentration spectroscopically at 600 nm. In addition to some protein-free HMW carbohydrate of molecular weight two million (or more), at least three other HMW fractions were present. Smaller molecular weight contaminants (eluting at a volume equal to the total column volume of 300 ml) contained both protein and carbohydrate. The single protein peaks indicated that either there are no HWM proteins (1-2 million daltons) or that protein enzyme degradation was efficient in this case. Evidence for the presence of a 1-2 million dalton protein in PF supernatants was indicated by comparing the chromatograms of supernatants before and after dialysis.



(a)



(b)

Figure 5. PF Extrudant Chromatograms before and after dialysis

Dialysis removed both protein and carbohydrates of less than 50 K daltons and revealed (in Figure 5 b) both 2 million molecular weight protein and carbohydrate in an approximate 2:1 mass ratio.

Fractions containing only HMW carbohydrate were collected from the column, dialyzed to remove salts and lyophilized affording 2.5 mg of white solid. Colorimetric assay of the solid indicated 0.41 mg of carbohydrate, 0.10 mg protein (4.3%) and 0.005 mg uronic acid (0.6%). Using the factor of

1.3 to convert total carbohydrate to total polysaccharide afforded 0.53 mg (21%) total polysaccharide. The remaining 1.9 mg of the sample was unidentified. In further attempts to characterize this white solid, we have obtained the infrared spectrum (IR) of this sample as well as that of Dextran, a two million molecular weight polysaccharide. Carbohydrates exhibit strong absorption bands at 1028 and 1085 cm^{-1} while protein absorb strongly at 1637 and 1548 cm^{-1} (Geesey and Bremer, 1991). Results appear below:

Sample	Principle Absorption Bands (cm^{-1})
Dextran	1018 (s), 1080(s),
PF. EPS	1026 (w). 1085 (w), 1410(m), 1564(s), 1653(s) 1271 (m) and 2860(m)

w=weak, m= medium, s= strong

Carbohydrate and protein were indicated by PF EPS spectra. Bands at 1271 and 2860 could have indicated phosphate and aldehydic groups respectively. The aldehyde group could have resulted from polymer chain cleavage. The absence of a band at 1604 cm^{-1} indicated that neither spectra contained significant water.

As a further test of purity, 1.8 mg of lyophilized PF HMW carbohydrate collected from the Sepharose 4B column was chromatographed on DEAE-Sephacel, an ion-exchange column. This material was eluted by a 0.010 M $KHPO_4$ buffer in 0.1 M NaCl. The NaCl concentration was increased linearly from 0.1 M to 1.0 M to elute all of the material. A single peak in

the chromatogram was evidence of the sample purity (Read and Costerton 1987). One hundred seventy fractions were collected. After the carbohydrate assay of a majority of these fractions, no significant amount of carbohydrate was found in any fraction. Assuming that the sample was 30% carbonydrate and that it eluded in a volume of 30 ml, one could expect the aliquot size used for analysis (0.5 ml) to produce an absorbance of 0.09. A reading of this magnitude was not significantly different from that of the blank. Apparently, not enough sample was placed on the column for a meaningful analysis.

Other attempts to determine the purity of HMW PF EPS eluting from Sepharose 4B column in the void volume were made. A void volume fraction was chromatographed on an Isco gradient HPLC employing a size exclusion column. Elution with a pH 6.6 phosphate buffer at flow rate of 1.5 ml/min yielded a single peak of retention time of 2.35 min. By comparison, soluble starch (molecular wt ~one million) eluted in 2.35 minutes while glucose (molecular weight = 180) did not elute (or was not detected by the 254 nm UV detector) even after 11 minutes. In order to see peaks in the chromatogram, the detector sensitivity was increased to 0.01 T. This resulted in a very unstable, drifting baseline. Further separations with HPLC were abandoned.

A chromatogram of partially-purified OSL 0031 is depicted in Figure 6. Absorbances at 260 and 490 nm can be used to monitor protein and carbohydrate (Williams and Wilson 1975), respectively. Although the Lowry colorimetric test for protein is more sensitive and specific, both Horan (1986) and Platt (1985) used absorbance measurements at 260 nm to follow protein eluting from a chromatographic column. Absorbance measurements are quickly and conveniently made at 260 nm. Since nucleic acids also absorb at 260 nm, absorbances at 260 nm may reflect both

protein and nucleic acids (DNA and RNA). For fractions showing large 260 nm absorptions, amounts of protein are quantitated via the Lowry method. From Figure 6, both carbohydrate and protein apparently elute in the void volume (~ 2 million molecular weight), intermediate size carbohydrates free of protein were present, and the smaller molecular weight solutes were primarily protein.

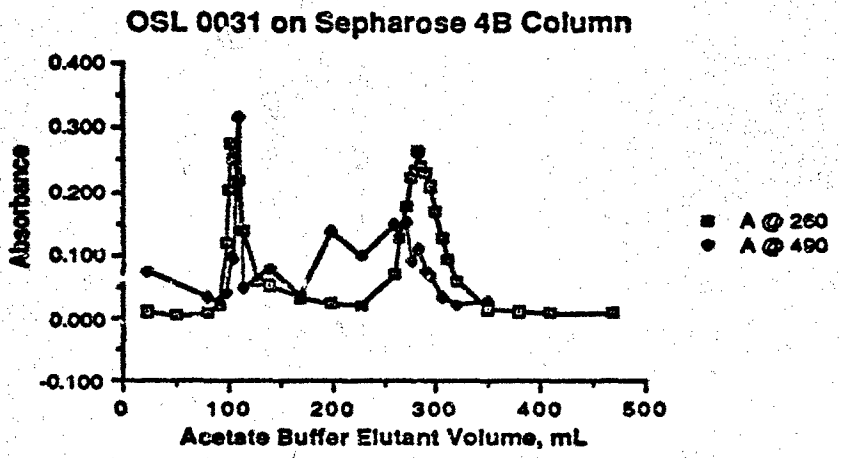


Figure 6.

Although Figures 7 and 8 show a relatively high absorbance at 260 nm at the column void volume, this absorbance was probably not due to protein. Colorimetric analysis of void volume fractions indicated the absence of protein and a very low concentration of DNA (2 µg/mL). Protein, carbohydrate and DNA colorimetric analysis of both HMW (fractions 20-30) and polymeric-MW fractions (85-100) are summarized (Table 2).

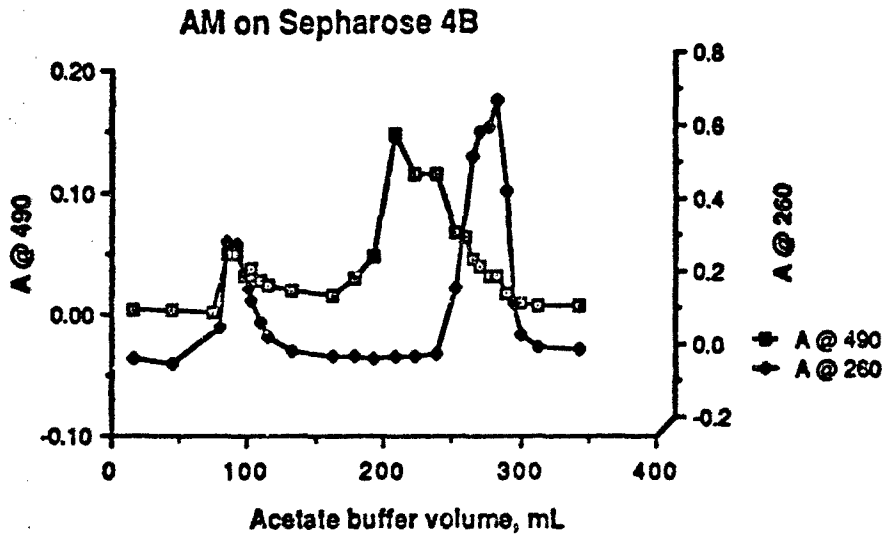


Figure 7

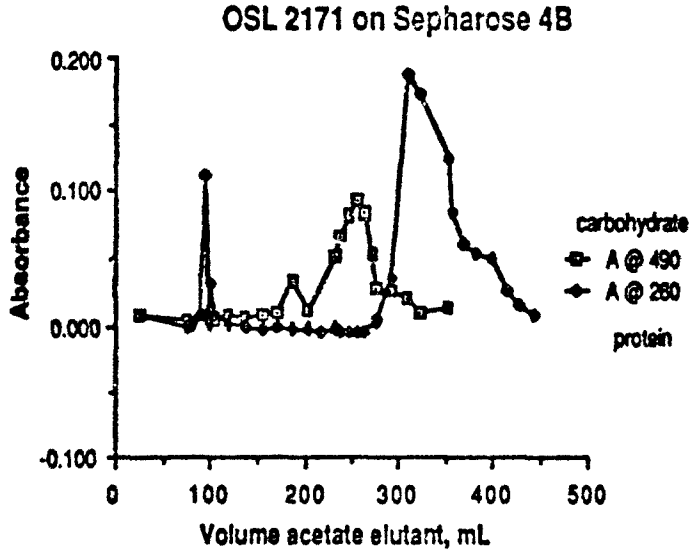


Figure 8

Table 2. Analysis of Chromatographic Fractions

Composition of High-Molecular- Wt Chromatographic Fraction (> 2 M)				
Sample	Fraction #	Composition (ug/mL)		
		Carbohydrate	Protein	DNA
PF	20-30	15	0	1.6
OSM	24-32	40	-	-
OSL 0031	28	90	-	-
	50-70	28	0	1.6
OSL 2171	20-28	0	0	-
AM	24	19	0	2.0

Composition of Polymeric Chromatographic Fraction (> 50 K)				
Sample	Fraction #	Composition (ug/mL)		
		Carbohydrate	Protein	DNA
PF	79-95	18	66(+6)	5.6
OSM	-	-	-	-
OSL 0031	80-100	31	80 (+13)	1.6
OSL 2171	78	29	0	-
	90	11	50	-
AM	90	14	170	6.8

The chromatograms of the various bacterial species and data in Table 2 show many similarities. No species showed protein components within the 2 million dalton fractions (fractions 20-30), indicating the efficiency of the protein degradation methods employed. In PF, AM and OSL 0031, HMW elutants were primarily carbohydrate with, at most, 10% DNA present. No correlation between a bacterial species HMW carbohydrate concentration and its growth rate or its absorbance after 100 hours (a measure of number of bacteria and concentration of polymeric metabolites) was noted. Later fractions (fractions 80-95) eluting from the size-exclusion column

had molecular weights smaller than 2 million but larger than 50,000. In most species these fractions were mostly protein with lesser amounts of carbohydrate and DNA.

During the summer of 1991, with the aid of SCWMRD personnel, we placed several formica plates in Charleston harbor and allowed a significant biofilm to develop on each. After retrieval and transportation back to the lab at SCSC, the biofilms were scraped off and extracted with distilled water. The water was filtered through a 4 micron filter, concentrated via rotary evaporator, and chromatographed on Sepharose 4B.

The resulting chromatogram below (Fig. 9) grossly resembled that of *F. fluorescens* supernatants, indicating the presence of HMW carbohydrate and small molecules of both carbohydrate and protein. The lab-produced bacterial extrudants were similar to those present in natural biofilms.

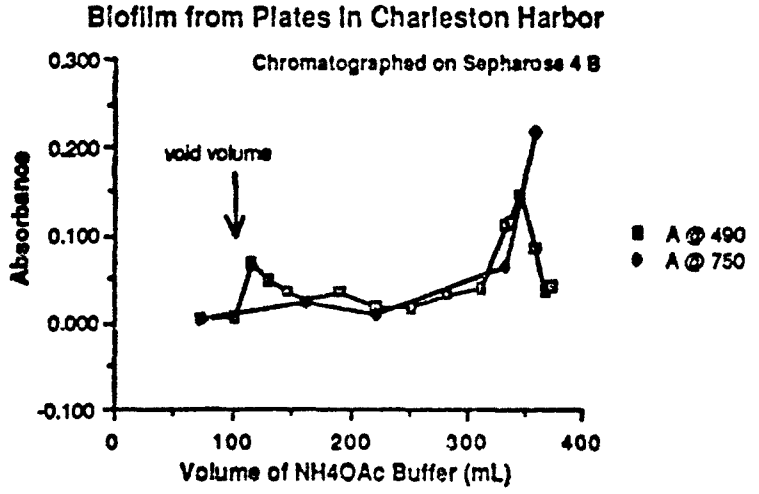


Figure 9.

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Characterization and Identification of Marine Bacteria Associated with Barnacles in Charleston Harbor, South Carolina

INTRODUCTION

Bacteria are microscopic, procaryotic, unicellular organisms that are found in all natural habitats on earth. Marine bacteria are an extremely important group of organisms that perform vital processes essential to life in the sea. Because marine bacteria initiate surface attachment (Sieburth 1979), and some bacterial species show a marked preference for attachment to submerged substrata (Tosteson et al. 1984), identification of these bacteria is important.

One of the long-term objectives of the SCSC-SCWMRD-ONR research project was to investigate biosensing parameters regulating bacterial and larval attachment on submerged substrata. Essential to understanding and controlling the molecular processes which govern the interactions among substrata, bacteria, and invertebrate larvae in marine environments (biosensing) is the identity of the organisms interacting. Knowledge of the characteristics, nutritional, physical, and chemical needs of the bacteria may provide information which will aid in the development of practical inhibitors for attachment of organisms to surfaces. This aspect of the research involved the characterization and identification of marine bacteria associated with barnacles in Charleston Harbor, South Carolina.

MATERIALS AND METHODS

A. Initial Work with Bacterial Samples in the SCSU-ONR Marine Microbiology Laboratory (MML)

When this investigator joined the research team in mid-November, 1991, refrigerated bacterial samples maintained in nutrient broth were available for study. Initially, oyster shells with attached barnacles, obtained in Charleston Harbor, SC, had been placed into buckets of ice and were brought to the laboratory in August, 1991. Each sample was named according to the substrate from which it had been obtained; for example, oyster shell, Rock No. 3. In the lab, sterile, cotton-tipped swabs were used to streak bacteria onto nutrient agar plates, which were incubated at 25 C for 24 to 48 hours. These plates contained mixed cultures of bacteria. Individual colonies from these plates were transferred to separate Erlenmeyer flasks containing 50 ml of nutrient broth. The oyster shells with attached barnacles were stored at -70 C.

The numerous flasks containing bacteria were sorted, and some bacteria from each flask were streaked onto Zobell's marine agar (ZMA) to determine colony purity. Some flasks contained pure cultures, and some did not. Pure cultures were separated, placed onto ZMA slants and allowed to grow for 18 to 48 hours at 25 C before they were subjected to a series of tests.

Several standard microbiological procedures and/or tests were performed in attempts to characterize and identify marine bacteria associated with barnacles attached to oyster shells. These included the negative stain, the Gram stain, catalase activity, gelatin hydrolysis, and the spot oxidase test (Baron and Finegold 1990, Case and Johnson 1984, Claus 1989, Kelley and Post 1989, Pelczar and Chan 1977, Seeley et al.

1991). Various procedures for the flagella stain (Claus 1989, Pelczar and Chan 1977, Presque Island Culture Company 1992) proved unsatisfactory, and flagella were not stained. After the above tests were performed, the oxidase positive bacteria were subjected to the Oxi/Ferm test, and the oxidase negative bacteria were inoculated into Enterotube II (Roche Diagnostic Systems, Inc. 1977, 1988).

The Enterotube II, when used with a computer coding and identification system (Roche Diagnostic Systems, Inc., 1988), incorporates 15 standard biochemical tests contained in a compartmentalized tube designed to permit the simultaneous inoculation of 12 test media, allows the identification of typical and atypical members of the Enterobacteriaceae . The ID value is a five digit number derived from a listing of the positive and negative reactions in Enterotube II for a given isolate. Similarly, the Oxi/Ferm tube test (Roche Diagnostic Systems/ Hoffmann-LaRoche Inc., 1977, 1988) is designed for the identification of oxidative-fermentative (O-F) Gram-negative rods. Nine biochemical tests are used. A computer coding and identification system permits the identification of specific organisms based upon the positive reactions. A coding manual is used to identify the organism. Where two or more organisms are listed under one identification value, additional confirmatory tests are needed to further separate the organisms. The ID value for the Oxi/Ferm system utilizes a four digit number to represent the test results. Various confirmatory tests such as susceptibility to certain antibiotics, wrinkled growth pattern, motility, growth at 42 C, etc. were utilized to confirm the identity of the bacteria. The API Rapid NF Test (Bio Merieux 1992) is another system which currently is being evaluated. It consists of 20 tests using the rapid NFT strip. Results are interpreted by use of the Rapid NFT data base. A seven digit code is used to identify the organism. For marine bacteria, the incubation time had to

be extended. These tests were designed for incubation at 37 C and often were/are used to identify pathogens in humans.

The cultures which were already in the SCSU-CNR MML were used to work out the general procedures used to identify marine bacteria. Additional samples from Charleston Harbor were obtained and analyzed.

B. Bacterial Plating from Natural Substrata...Winter, 1992

During low tide, midFebruary, 1992, oyster shells containing living barnacles, *Balanus improvisus*, were collected from a large rock behind Grice Marine Biological Laboratory in Charleston Harbor. These substrata were taken into the laboratory for plating onto six different types of agar. Swabbings from two oyster shells and barnacle tests were used. These plates were designated OSM=oyster shells from the mid-intertidal region. Other swabbings were taken from two oyster shells and empty barnacle tests from the low intertidal region on the southwest side of the point. These oysters were growing in a free-standing clump and had a couple of empty *B. improvisus* tests attached to them. These were labeled OSL=oyster shells from the low intertidal region . Also swabs were taken from an artificial substratum (formica plate) which had been suspended in the low intertidal region above the mudflat. This plate had been outside since mid December. Plated material from the grooved (rough) side of the plate was used. These plates were designated ASL=artificial substratum, low intertidal region.

Bacteria from the OSM, OSL, and ASL substrata were plated onto duplicate plates of eosin methylone blue agar (EMB), pseudosel agar (PA), photobacterium medium (PBM), Zobell's marine agar (ZMA), mannitol salt agar (MSA), and thiosulfate citrate bile sucrose medium (TCBS). The plates were put into a covered cardboard box and were stored overnight at

27 C. The plates were transported from Charleston to the Orangeburg SCSU-ONR MML where they were incubated for 24 hours at 25 C. These plates were stored in the refrigerator until they could be further observed and prccessed.

Bacteria were transferred from each medium into separate 0.5 mL aliquots of marine salts diluent (MSD) and were streaked onto Zobell's marine agar, incubated at 25 C for l8 to 24 hr. (or 48 hr. for slow growing colonies) before specific tests were performed.

C. Bacterial Plating from Natural Substrata...Summer, 1992

A mid June sampling of the bacteria associated with barnacles and oyster shells was taken. Samples were taken from both OSM and OSL areas. In addition to the six types of media mentioned in part B, nutrient agar plates were used. In the field, samples were streaked in triplicate, brought back to the SCSU-ONR MML and incubated at 25 C for l8 hours. For each colony type present, a dilution using 0.5 ml of marine salts diluent was made. This cell suspension was then streaked onto ZMA plates and were incubated at 25 C for l8 to 48 hours. Each plate was labeled according to the original natural substratum and medium. Results were observed and recorded. The isolated colonies were subjected to the battery of standard tests (part A). Specific tests performed for some of the bacteria in this group were the motility, penicillin, sucrose, anaerobic growth tests and growth on MacConkey medium.

RESULTS

A. Initial Work with Bacterial Samples in the SCSU-ONR Marine Microbiology Laboratory

Analysis of five separate nutrient broth cultures of bacteria associated with the barnacle, *Balanus eburneus* , attached to Rock No. 3 revealed both Gram positive and Gram negative bacilli. Visually three types of colonies developed: creamy, orange, and yellow. The yellow colony from sample four was identified as group 5 A-2 *Pseudomonas* like/*Pseudomonas cepacia/Pseudomonas fluorescens/Achromobacter* species Biotype 2. The Oxi/Ferm ID value was 0071. Enterotube II test results for the orange and yellow colonies associated with Rock No. 3, sample three--ID value 20000-indicated *Shigella* serogroups A, B, or C, */Enterobacter agglomerans* . The creamy colony having an ID value of 20103 was not identified.

Pure cultures have been isolated, but many of them have not been identified and/or additional confirmatory tests need to be performed. As indicated previously, these samples were mainly used to teach students technique and to work out procedures which would help in the identity of marine bacteria.

B. Bacterial Plating from Natural Substrata, Winter, 1992

OSM:

Colonies transferred from the original plating medium to ZMA gave the following results when inoculated into **Oxi/Ferm**. The color of the colonies on the original medium is indicated.

OSM:

Colony Color	ID Value	Identity
EMB Pink	2031	*Pseudomonas fluorescens*
EMB Light Purple	1151	*Pseudomonas stutzeri*
EMB Blue	1121	*Achromobacter* species
TCBS Dark Green	4001	*Flavobacterium* sp./ *Pastuerella haemolytica*/ *P. ureae*
MSA Yellow	0001	*Pseudomonas species*/ *Alcaligenes faecalis*/ *Pasteurella ureae*/ *Moraxella species*/ Group 2K-I *Pseudomonas-Like*
EMB dark Blue	0071	Group 5A-2 *Pseudomonas-like*
EMB Light Purple	2071	*Pseudomonas fluorescens*/ *P. pseudomallei*/ *Achromobacterspecies* Biotype 2
TCBS Orange	0201	Group 2F *Flavobacterium-like*/ *Pasteurella multocida*
TCBS Dark Green	4041	*Vibrio alginolyticus*/ *Flavobacterium species*/ *Aeromonas hydrophila*

OSL:

Colony Color	ID Value	Identity
EMB Purple-Creamy	0031	*Alcaligenes faccalis/ Pseudomonas species/ Group 4E Alcaligenes-like/ Group M-4f MORAXELLA-LIKE*
EMB Dark Blue	2171	*Pseudomonas aeruginosa/ P. putida/ P. fluorescens*
EMB Purple=White	2031	*Pseudomonas aeruginosa/ P. fluorescens/ Achromobacter species Biotype 2*
EMB Swarmy	2171	*Pseudomonas aeruginosa/ P. putida/ P. fluorescens*

ASL:

EMB Pink	0471	No ID.

Colonies from the artificial substrate were not processed beyond the initial standard tests, i.e., were not subjected to the Oxi/Ferm or Enterotube II tests.

C. Bacterial Plating from Natural Substrata..Summer, 1992

The following bacteria from oyster shells in the mid-intertidal zone were identified and confirmed by additional tests (Kreig and Holt 1984; Roche Diagnostic Systems, Inc., 1977, 1988).

OSM:

Colony Color	ID Value	Identity
ZMA White, Swarmy	4211	*Vibrio parahaemolyticus*
Nutrient Agar, Peach	2010	*Enterobacter agglomeran*
ZMA White	4241	*Vibrio alginolyticus*
PBM, White, Swarmy	4241	*Vibrio parahaemolyticus*
ZMA, Yellow	0001	*Moraxella species*

DISCUSSION

Basic microbiological tests were utilized in attempts to characterize and identify marine bacteria associated with rocks, oyster shells and barnacles found in the low and mid-intertidal zones in Charleston Harbor, South Carolina. Specific biochemical tests as found in the Enterotube II and Oxi/Ferm tests and in *Bergey's Manual* (Krieg and Holt 1984) were helpful in further characterizing and/or identifying these bacteria. The API Rapid NF test is being evaluated for its use in the identification. Both the Oxi/Ferm and Enterotube II tests were designed for organisms which were to be incubated at 37 C. For marine bacteria, the temperature was decreased to 25 C, and the incubation time had to be increased from two days to five to seven days.

In some samples, both Gram positive and Gram negative bacteria were found, but for tests other than the basic, general ones, the Gram negative, oxidase positive or negative bacteria were processed because the above mentioned tests were designed to identify Gram negative bacteria. Moreover, reports in the literature indicate that the majority of marine bacteria are Gram negative (Zobell 1944, Rheinheimer 1981). Most of the

media used in this investigation selected for Gram negative bacteria. The marine bacteria observed were very small and exhibited, in some instances, a gradation of colors. This resulted in the same chemical reactions and/or identity.

Additional tests need to be performed to separate organisms which are placed into the same group, i.e., have the same ID value or computer code. Also investigation of bacteria which give results for which there are not computerized ID values or codes in the Enterotube II and Oxi/Ferm codebooks should be performed. The characterization and identification of bacteria obtained from the summer 1992 OSL region needs to be completed, and an evaluation of organisms found in each region needs to be done. Moreover, a recheck and confirmation of the purity and/or integrity of the cultures identified needs to be performed before these cultures are put into the SCSU-ONR stock culture collection. Stock cultures are stored in glycerol at -70 C. Stock cultures will be available for use by other laboratories at their request. Information derived from this study will be compiled into a manuscript and submitted to the Journal of Applied and Environmental Microbiology.

CONCLUSION

Standard microbiological procedures and/or tests have been used to identify and characterize bacteria associated with oyster shells and barnacles in both the low and mid-intertidal zones in Charleston Harbor, South Carolina. The use of *Bergey's Manual*, Enterotube II , Oxi/Ferm, and various confirmatory tests have resulted in the identity of numerous Gram negative bacilli and coccobacilli. The identities of some bacteria which did not produce codes compatible with those in the computerized databases of the Oxi/Ferm and Enterotube II tests need to be determined.

Further investigation, especially of the bacteria found in the low intertidal zone, needs to be done. Confirmation of the identity of cultures from the winter and summer sampling needs to be performed before these cultures are added to the stock pure culture collection, stored in glycerol at -70 C, in the SCSU-ONR MML.

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LARVAL ATTACHMENT BIOASSAYS

INTRODUCTION

Microbial films on submerged surfaces, especially films of attached bacteria, tend to stimulate settlement behavior of larvae from a variety of invertebrate phyla (reviews: Meadows and Campbell 1972; Scheltema 1974; Crisp 1984; Bonar et al. 1986; see also, Weiner et al. 1989; Tamburri et al. 1992). However, inhibition of larval settlement by bacteria has been reported rarely. The chemical inducers of settlement derived from bacteria tend to be soluble in seawater (Neumann 1979; Fitt et al. 1989, 1990; Tamburri et al. 1992) and small in molecular weight (Fitt et al. 1990; Tamburri et al. 1992).

In contrast to results reported for other invertebrates, most bacterial species tested so far either have no effect on or inhibit settlement (defined here as permanent, attachment to a surface) of barnacle cypris larvae. Maki et al. (1988) tested the effect of 18 different species of bacteria attached to polystyrene substrata on settlement of cypris larvae of the barnacle *Balanus amphitrite*. Of the 18 species, seven were inhibitory, one was stimulatory, and ten species had no significant effect on larval attachment. The gram-negative bacilli, *Deleya marina* and *Alteromonas macleodii*, cultures which were originally isolated from seawater, were two of the species that most inhibited attachment. Films of *D. marina* that had been aged on polystyrene surfaces were more inhibitory to attachment of *B. amphitrite* than newly-established biofilms (Maki et al. 1988, 1990). However, *D. marina* tended to stimulate attachment of *B. amphitrite* on glass surfaces. Exopolymers produced by *D. marina* in culture both inhibited and stimulated attachment of *B. amphitrite* on polystyrene, depending on the degree to which they were

diluted in the assay (Maki et al. 1990). In similar bioassays, Holmstrom et al. (1992) found that 5 of 40 bacterial isolates from subtidal surfaces inhibited attachment of *B. amphitrite* larvae. One isolate was studied in more detail. When aged up to 5 days, biofilms of the isolate inhibited larval attachment, but biofilms aged 6 days had no effect on attachment. This particular isolate when cultured released a small molecular weight component that was toxic to the larvae.

It is clear that substratum-bacterial-larval interactions are very complex. A major problem is that the nature of the molecular components of bacterial exopolymers is not well understood, and their chemical structures and physical interactions with substrata need to be examined in considerable detail. In addition, prior studies of larval barnacle responses to bacteria have utilized only one species of barnacle, *Balanus amphitrite*, in attachment assays. The behavior of this species during settlement, and its patterns of attachment, might not be representative of balanid species as a whole. Our ONR-supported research was designed to address this problem.

An objective of the original proposal was to test the settlement response of larvae of the common estuarine *Balanus eburneus* to films of bacteria on substrata, focusing on the gram-negative bacterium *Pseudomonas fluorescens*. However, preliminary work was necessary to determine whether the responses of this barnacle species were suitable for use in laboratory attachment bioassays and representative of other barnacle taxa. Therefore, a series of experiments were devised to compare the attachment characteristics of larvae of three barnacle species (*B. eburneus*, *B. amphitrite*, and *B. improvisus*) to polystyrene and glass substrata typically used in attachment bioassays.

Summary of Larval Bioassay Research

A system for rearing larvae of three barnacle species has been established at Marine Resources Research Institute (MRRI) using culture procedures modified from those of Ritz et al. (1992). Batches of larvae were produced for use in laboratory bioassays that were similar in development rate and settlement behavior. Adult broodstock were collected every 2-3 months from local habitats, and maintained in the laboratory. Larvae were generally available year-round, although larval availability and survival were greatest in autumn through spring. Bacterial culturing was also performed at MRRI.

Following the general procedures of Rittschof et al. (1992) and Maki et al. (1988), we devised a bioassay system for monitoring the attachment of cypris stage larvae of the barnacles *Balanus amphitrite, B. improvisus,* and *B. eburneus*. Briefly, cyprids of identical ages were introduced in groups of approximately 25 to polystyrene Petri dishes or borosilicate glass vials that were either sterile or contained films of bacteria attached to their surface. Attachment of cyprids were then monitored daily for 4-7 days, while assay containers were maintained at constant conditions of 26 C (± 1 C) and 4 h light: 10 h dark.

A comparative study of larval attachment of the three test barnacle species to polystyrene and glass substrata without bacteria was completed and presented at the 1992 Crustacean Society Meeting (Appendix I). Results of the study indicated that, for all three barnacle species, attachment after one day was low, even when larvae were "aged" (stored at 6 C for 1 to 3 days) prior to use in the assay. The aging of cyprids was thought to increase their attachment (Branscomb and Rittschof 1984). After 4 days, 60 -90% of larval *B. amphitrite* and *B. improvisus* attached to both substrata, although *B. amphitrite* tended to

attach to glass in greater numbers than to polystyrene. *B. improvisus* tended to prefer polystyrene substrata. *Balanus eburneus* attached in very low numbers to both polystyrene and glass, and aging the cyprids tended to decrease subsequent attachment. Aging had no effect on attachment of *B.amphitrite*, and slightly decreased attachment of *B. improvisus*, especially on glass substrata. It was concluded that *B. eburneus* was not a good model organism for use in this type of attachment bioassay and that *Balanus* spp. do not conform in settlement behavior to one model. We plan to assemble this information into a manuscript for submission to the Journal of Experimental Marine Biology and Ecology.

In addition, the influence of three species of bacteria (*Deleya marina*, *Alteromonas macleodii*, and *Pseudomonas fluorescens*) on attachment of cyrpris stage larvae of the barnacles *Balanus amphitrite* and *B. improvisus* was examined. Bacteria grown in culture to midexponential or stationary growth phase, or their extracellular materials (ECM) produced during culturing, were exposed to sterile polystyrene Petri dishes (Falcon 1006, 50 x 9 mm) or muffled borosilicate glass vials (27 mm diameter). Five milliliters of washed bacterial cells (approximately 1 x 109 cells/ml), ECM solution, or sterile-filtered (0.2 µm) seawater (FSW) were added to the dishes or vials and the material was allowed to attach for 2 or 4 hours. Substrata were gently rinsed free of unattached material, then the dishes or vials were refilled with 4.5 ml of FSW and approximately 25 larvae that recently molted to the cypris state. Attachment of cyprids to each dish was monitored at 24 (+2) hours intervals for at least four days, to monitor changes in attachment behavior of cyprids over time. Assays were terminated by rinsing unattached cyprids from the substrata with distilled water, determining the number of attached and unattached cyprids, then calculating the

proportion that attached during each day of the assay. Assays having larval mortality greater than 30% were not used in calculations.

The mean proportion of larvae attaching on each day was calculated for each treatment (control, bacterial cells, bacterial ECM), and the data tested for normality and homogeneity of variances. Differences among the treatments in the mean proportion of larvae attaching over time were statistically analyzed using multivariate and repeated measures analysis of variance techniques.

Experimental data revealed that the variability in attachment of larvae within each treatment of an assay is high. Because of this variability, the number of replicates in each treatment was increased from three (the number used by Maki and Rittschof) to six to increase the power of the statistical analyses. In addition, each experiment was repeated at least three times to determine the reliability and consistency of results.

Results of assays using stationary phase cells and ECM indicated that *Deleya marina* cells and ECM on polystyrene surfaces retarded attachment of larval *Balanus amphitrite*, but mixed results (both stimulation and retardation) were observed on glass substrata. *Alteromonas macleodii* cells and ECM stimulated larval attachment to polystyrene and glass. *Pseudomonas fluorescens* cells and ECM had mixed effects on larval attachment. Within an experiment, results tended to be consistent over time (up to 1 week), but results were not consistent from one experiment to the next. In addition, bacterial cells and ECM did not always produce similar effects on larval attachment. These results were presented at Benthic Ecology Meeting on April, 1-4 1993 (Appendix II).

The effects of bacterial cells and ECM from stationary phase cultures on attachment of larval *Balanus improvisus* were also examined. Results

of attachment bioassays with *Deleya marina* and *Pseudomonas fluorescens* were similar to those observed for *B. amphitrite*. However, *Alteromonas macleodii* cells and ECM retarded attachment of larval *B.improvisus* to polystyrene. This is in contrast to the stimulation of attachment seen for *B. amphitrite*.

Additional bioassays testing the effects of mid-exponential phase bacterial cells and ECM on attachment of *Balanus amphitrite* were performed. *Deleya marina* and *Pseudomonas fluorescens* produced effects on larval attachment similar to the results for stationary phase cells and ECM. However, *Alteromonas macleodii* cells and ECM from mid-exponential cultures retarded larval attachment to polystyrene, a result different from that observed when stationary phase cells and ECM were used in the bioassays.

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Appendix I

Comparative Larval Settlement Behavior In Three Species Of Barnacle (*Balanus amphitrite, B. eburneus and B. improvisus*)

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INTRODUCTION

Recently, *Balanus amphitrite* has become the "white rat" of barnacle settlement research, by providing a model system for studies of larval attachment. However, its settlement response may not be representative of that of other balanomorph species. We initiated the project described here to determine whether larval *B. eburneus* and *B. improvisus* respond to surfaces during settlement in still water conditions in a manner similar to *B. amphitrite*. If they do, then these species too could be used in assays of responses of larvae to environmental factors.

METHODS

Larvae were reared to the cypris stage in laboratory cultures. Cyprids were removed from culture the day they appeared (=Day 0) and used immediately, or stored at 6 C until experimentation.

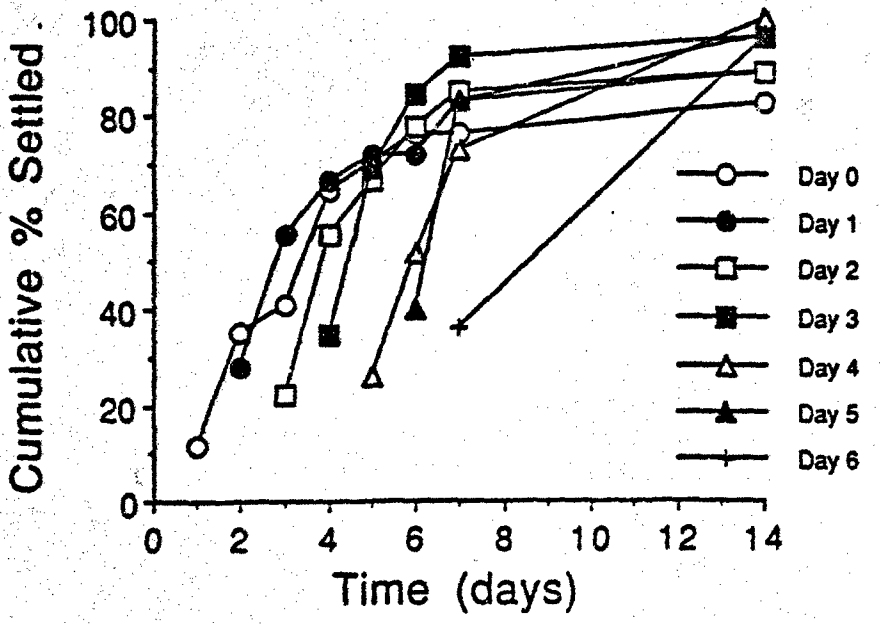
Settlement assays were performed in polystyrene petri dishes and borosilicate glass vials. Groups of approximately 20 - 50 cyprids were placed in filtered seawater in each dish or vial. Settlement was monitored daily, and settled cyprids and metamorphosed juveniles were marked in red.

Question: What is the settlement response of larvae of the three species over time, and does the response vary with larval age or substratum type?

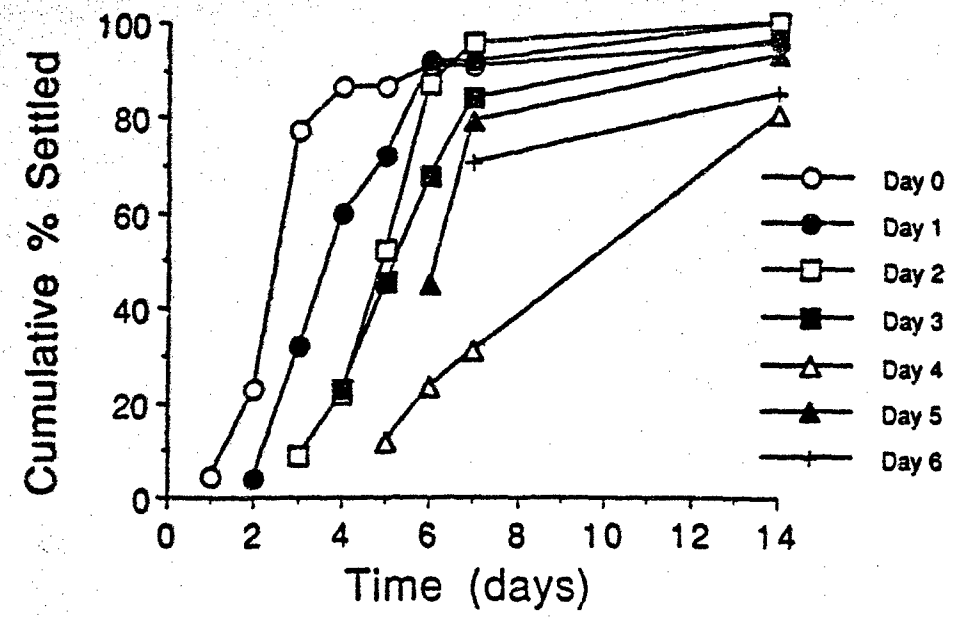
Approach: Monitor settlement of cyprids of various ages (Day 0 - Day 6) onto polystyrene and glass substrata over time.

Results: *Balanus amphitrite* and *B. improvisus* cyprids settled consistently in high numbers, whereas *B. eburneus* settled in low numbers and settlement rate differed among batches of larvae. *B. improvisus* tended to settle in higher numbers on polystyrene, whereas *B. amphitrite* tended to prefer glass substrata. Settlement of *B. eburneus* cyprids tended to be lower the longer they were aged at 6 C.

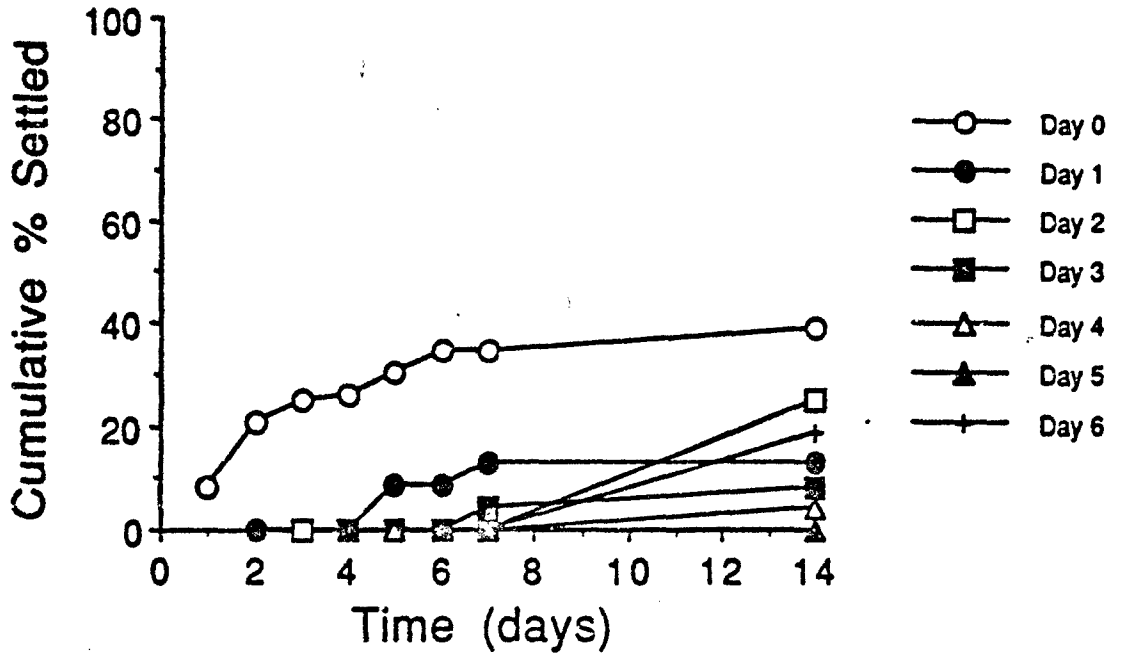
B. amphitrite #49 (polystyrene, 30 ppt)



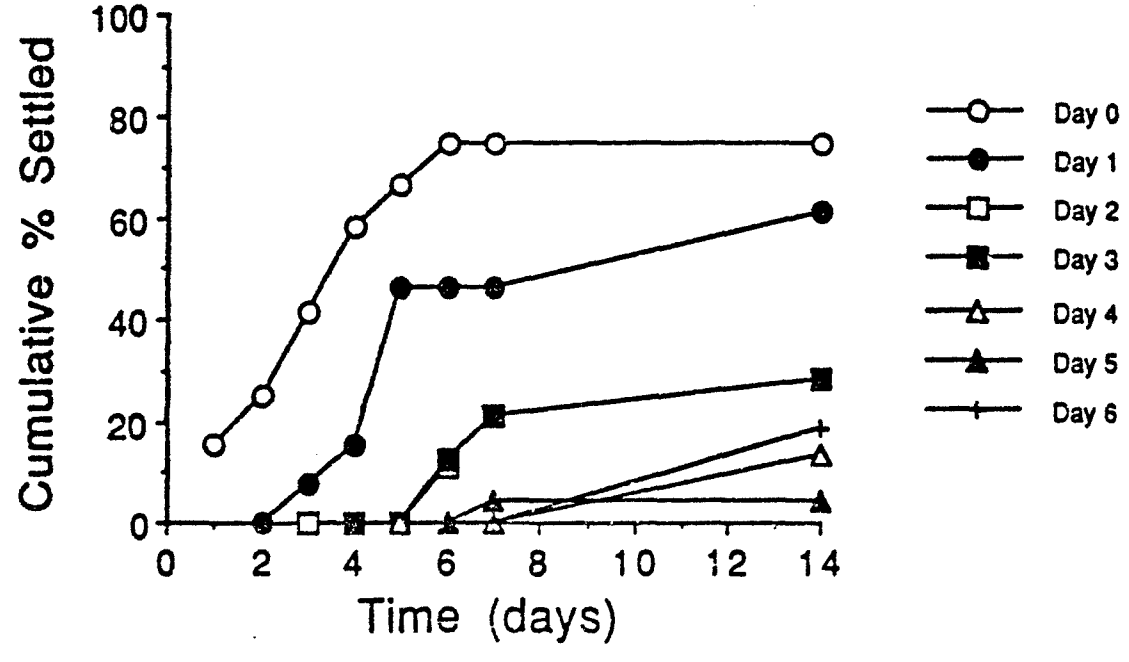
B. amphitrite #49 (glass, 30 ppt)



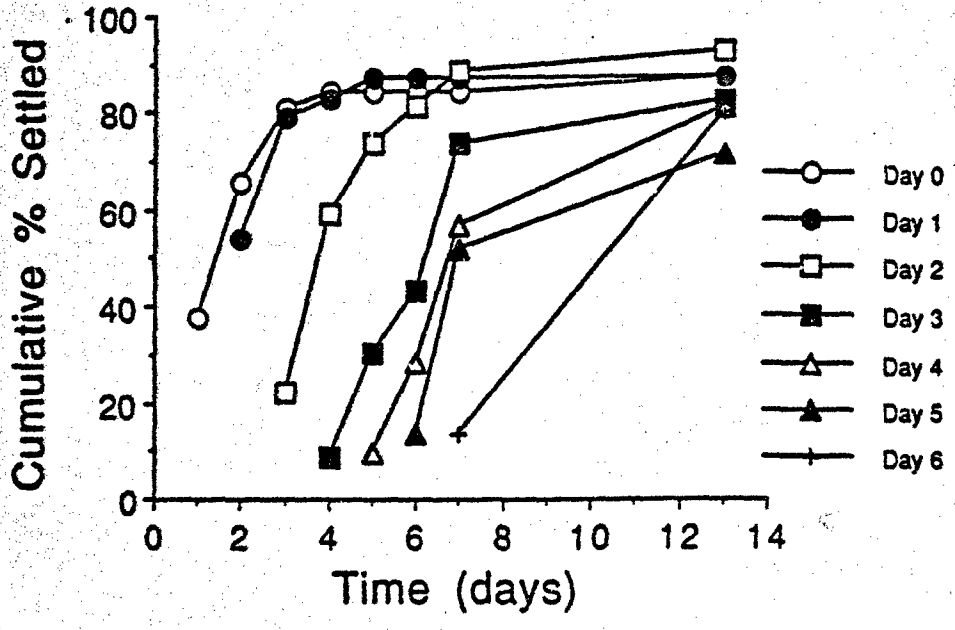
B. eburneus #61 (polystyrene, 30 ppt)



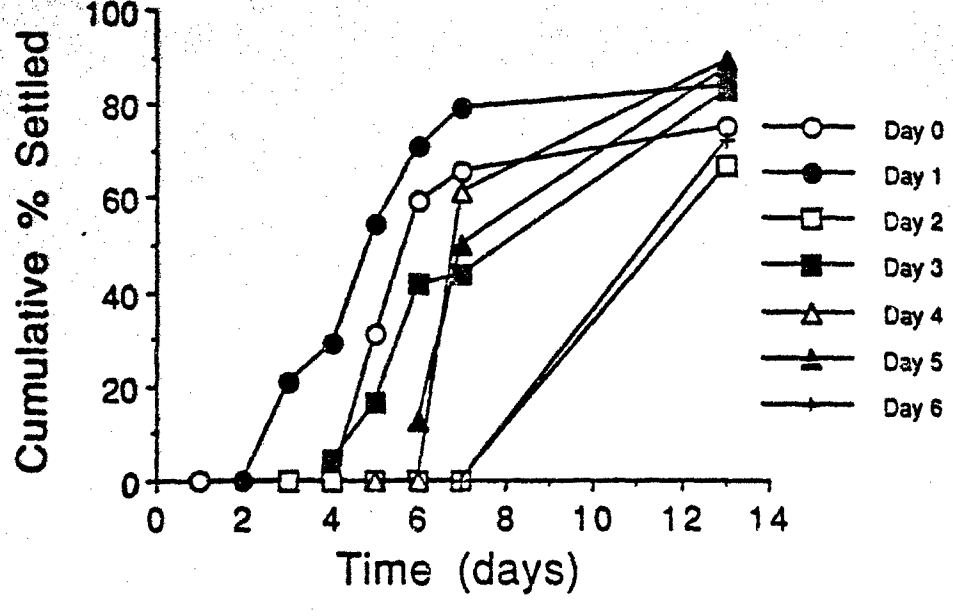
B. eburneus #92 (glass, 30 ppt)



B. improvisus #15 (polystyrene, 15 ppt)



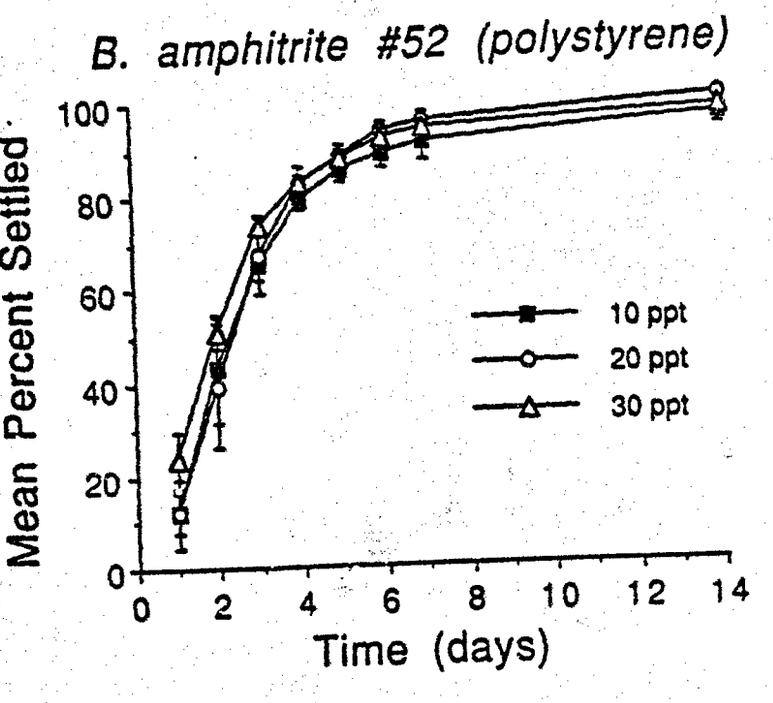
B. improvisus #15 (glass, 15 ppt)

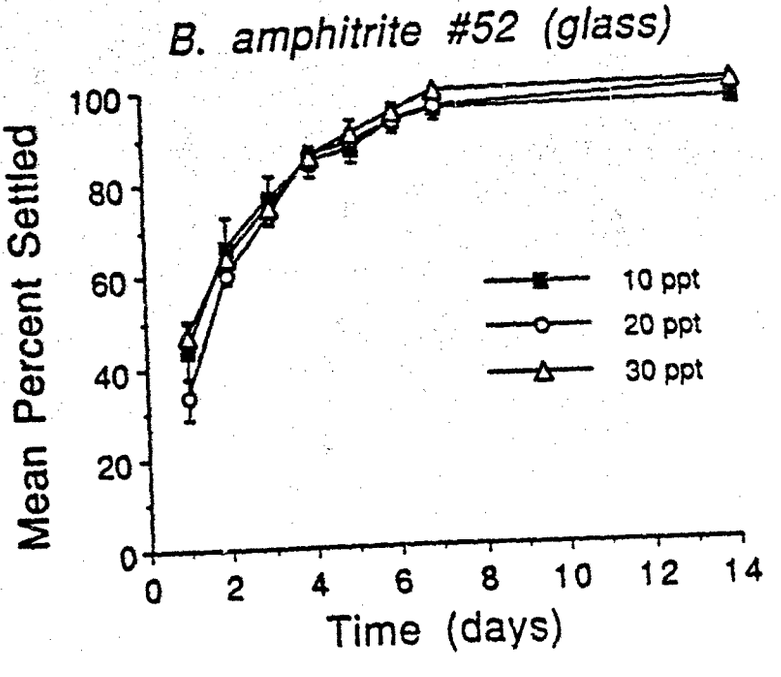


Question: Does the salinity of the seawater in the assays affect the number of larvae settling?

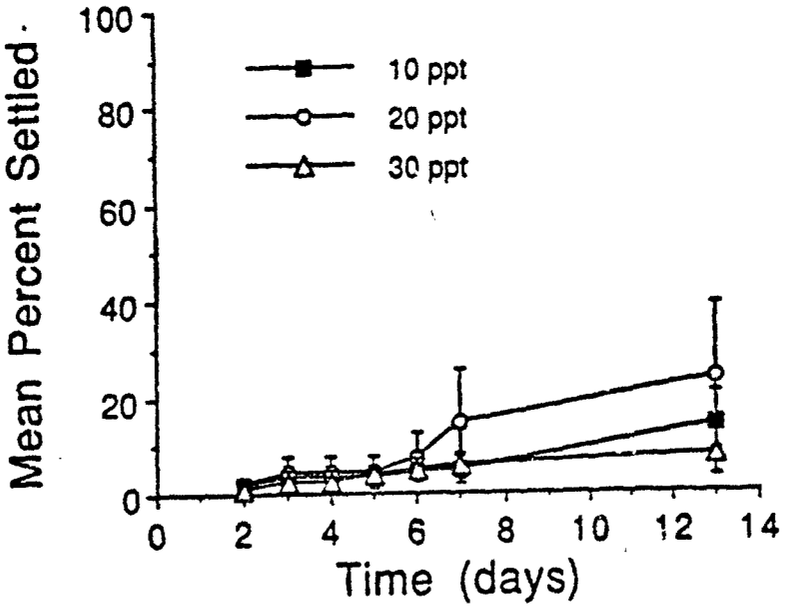
Approach: Day 0 cyprids were placed into each of 4 dishes or vials containing 10, 20, or 30ppt seawater.

Results: Salinity had less of an effect on settlement of *B. amphitrite* (usually found in high salinity areas) and *B. improvisus* (usually found in low salinity areas <15ppt) than expected. The effect of salinity on settlement of *B. improvisus* was greatest on glass substrata. Settlement of *B. eburneus* was very variable at all salinities.

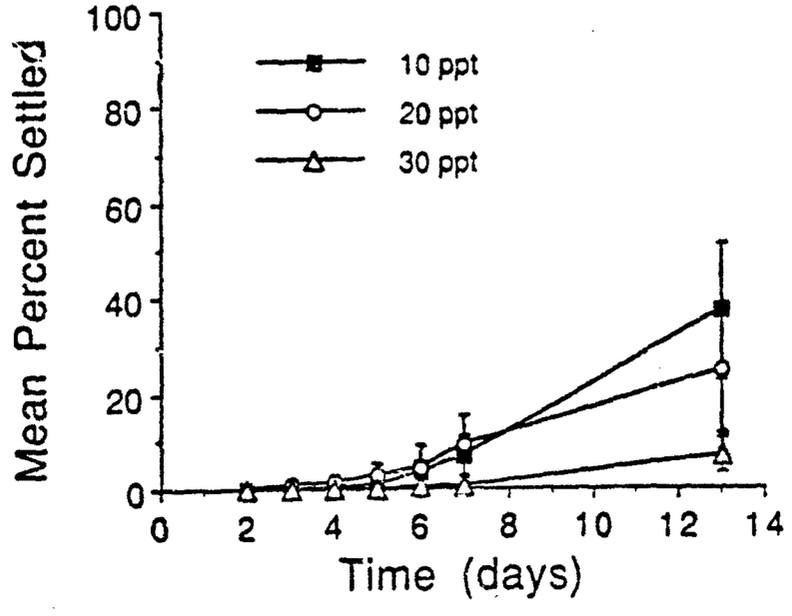

B. amphitrite #52 (polystyrene)
Mean Percent Settled
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (days)
10 ppt
20 ppt
30 ppt


B. amphitrite #52 (glass)
Mean Percent Settled
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (days)
10 ppt
20 ppt
30 ppt

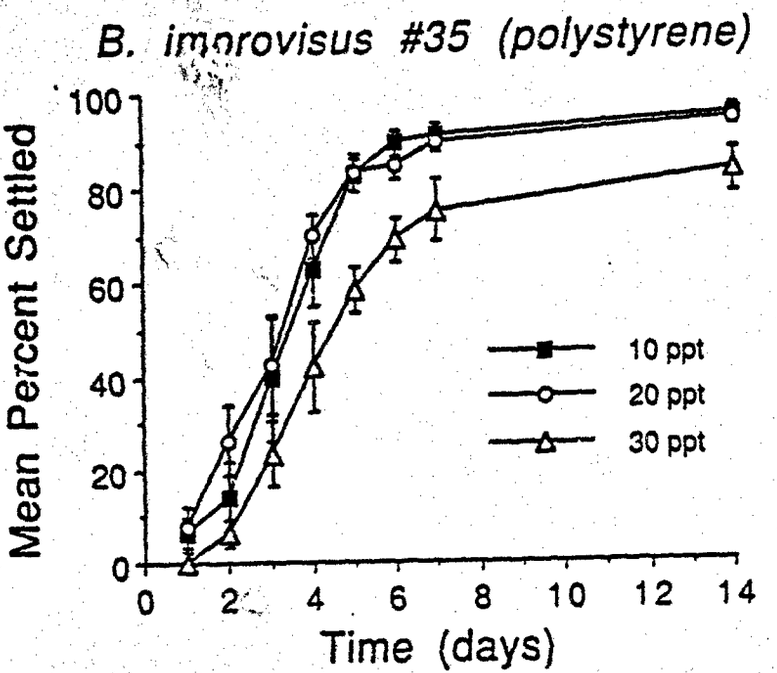
B. eburneus #115 (polystyrene)

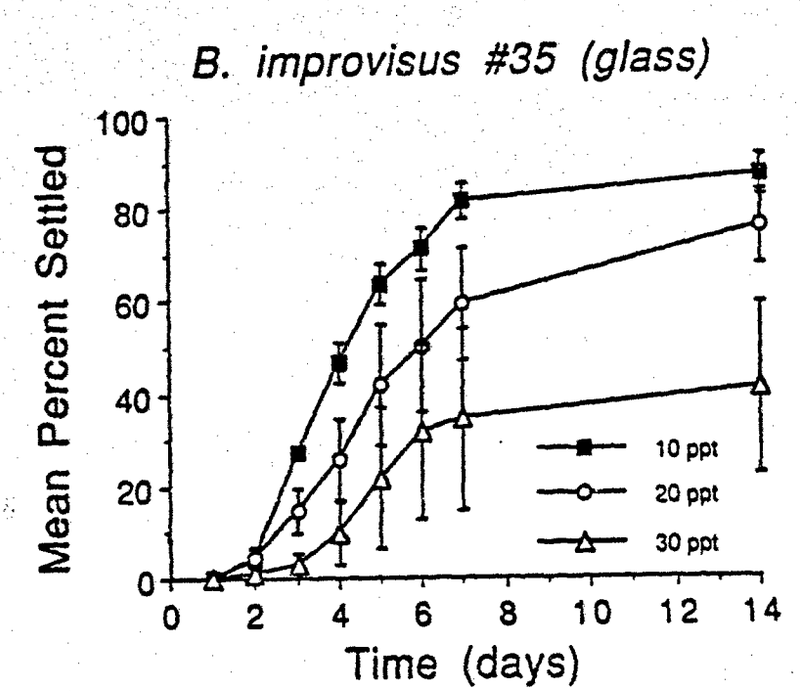


B. eburneus #115 (glass)



Alteromonas macleodii


B. improvisus #35 (polystyrene)
Mean Percent Settled
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (days)
10 ppt
20 ppt
30 ppt

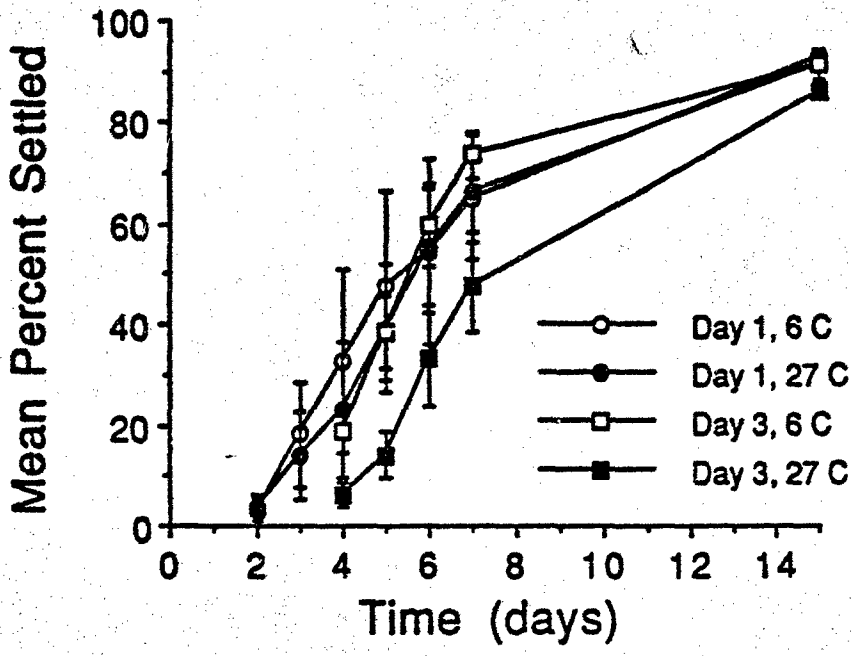

B. improvisus #35 (glass)
Mean Percent Settled
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (days)
10 ppt
20 ppt
30 ppt

Question: In many experiments on larval barnacle settlement, cyprids are "aged" by storing at low temperatures prior to experimentation. Does cooling the cyprids during storage affect subsequent settlement?

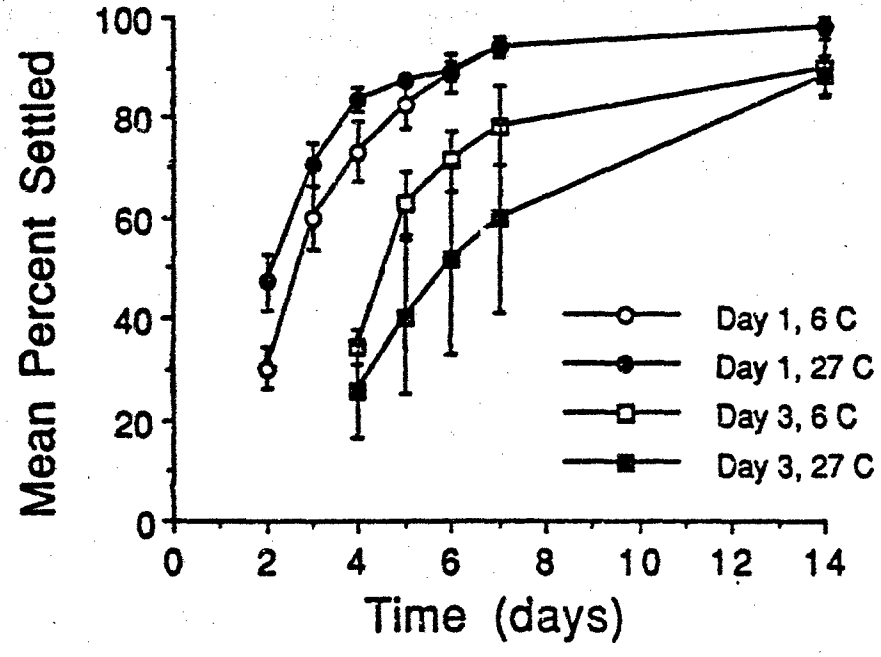
Approach: Day 0 cyprids were divided into two groups and stored at 6 C or 27 C until placed in experimental containers.

Results: Cooling cyprids prior to experimentation had: 1) no effect on settlement of *B. amphitrite*; 2) an inconsistently negative effect on settlement of *B. eburneus*; and 3) a slight negative effect on settlement of *B. improvisus*, especially on glass substrata.

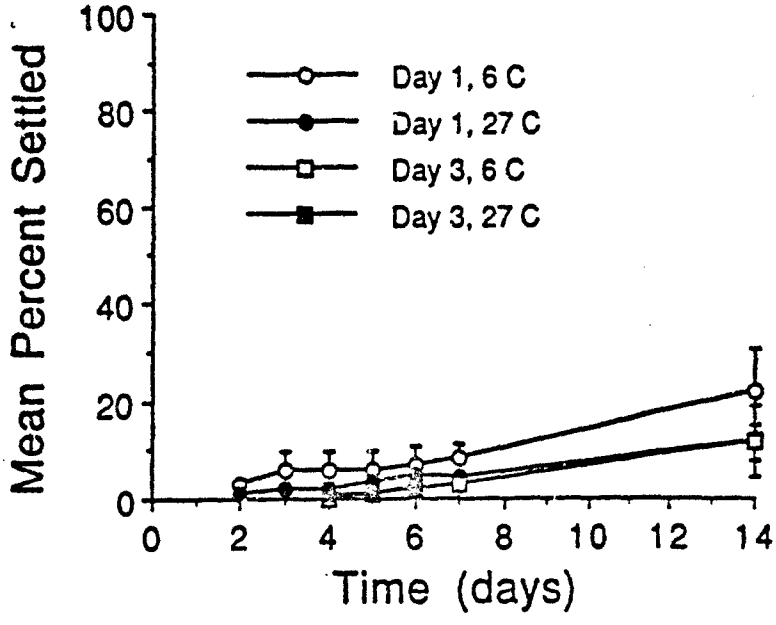
B. amphitrite #50 (polystyrene, 30 ppt)



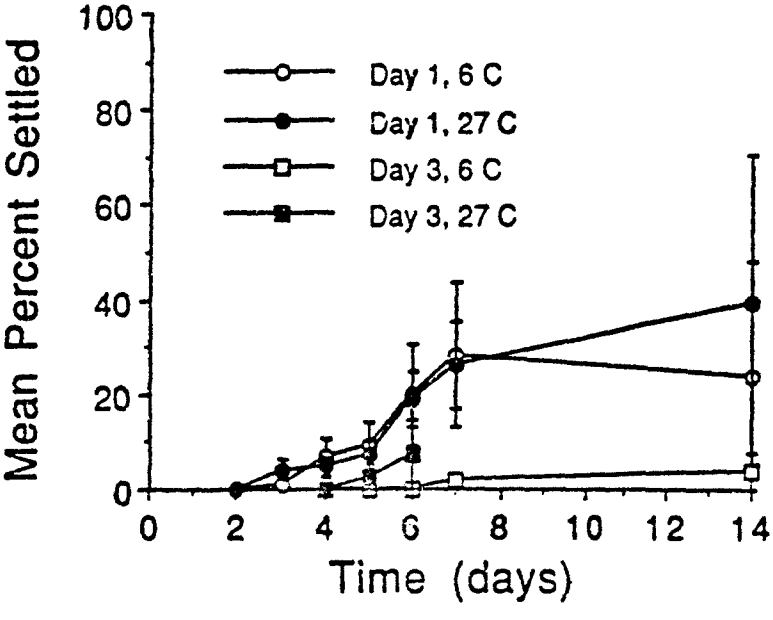
B. amphitrite #55 (glass, 30 ppt)



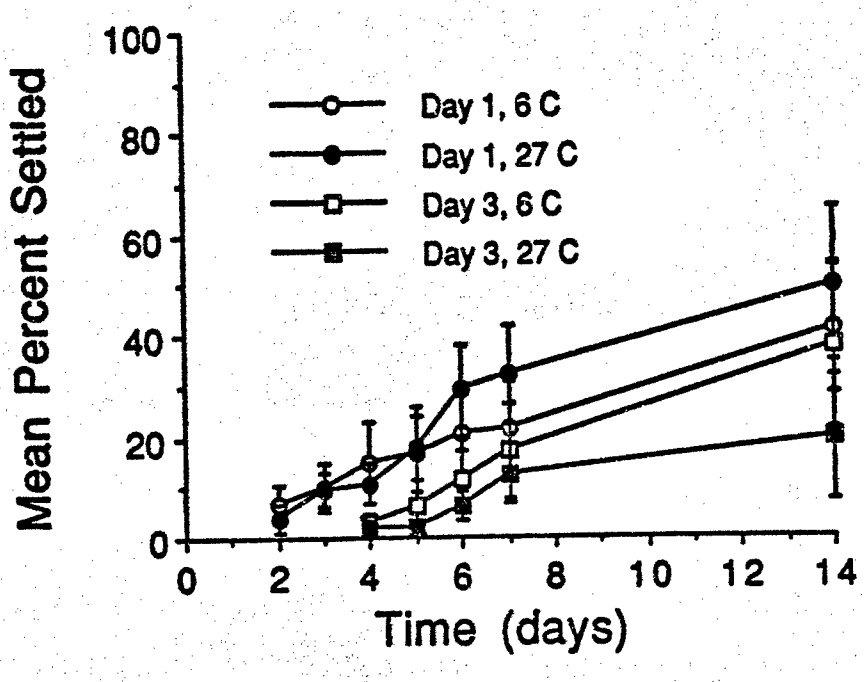
B. eburneus #106 (polystyrene, 30 ppt)



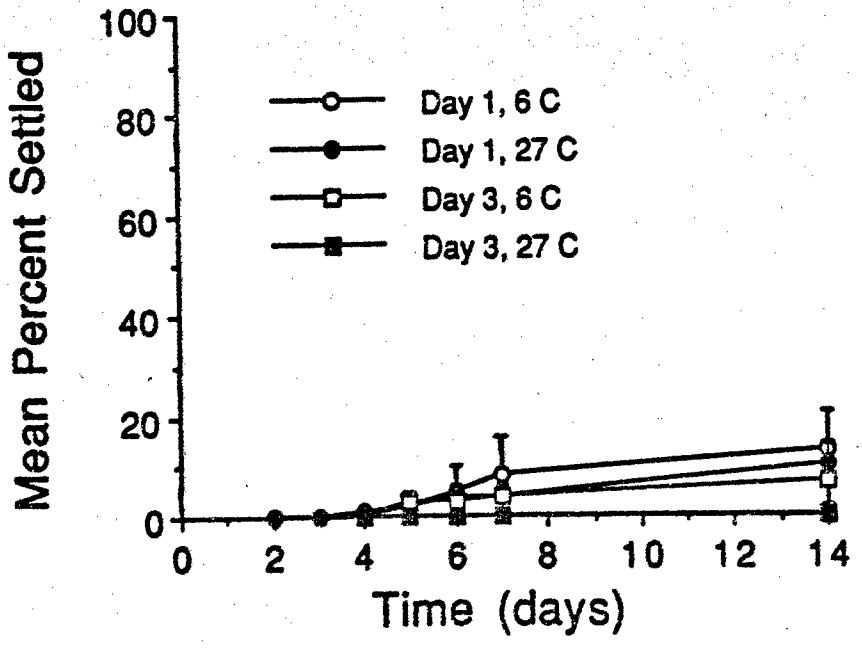
B. eburneus #106 (glass, 30 ppt)



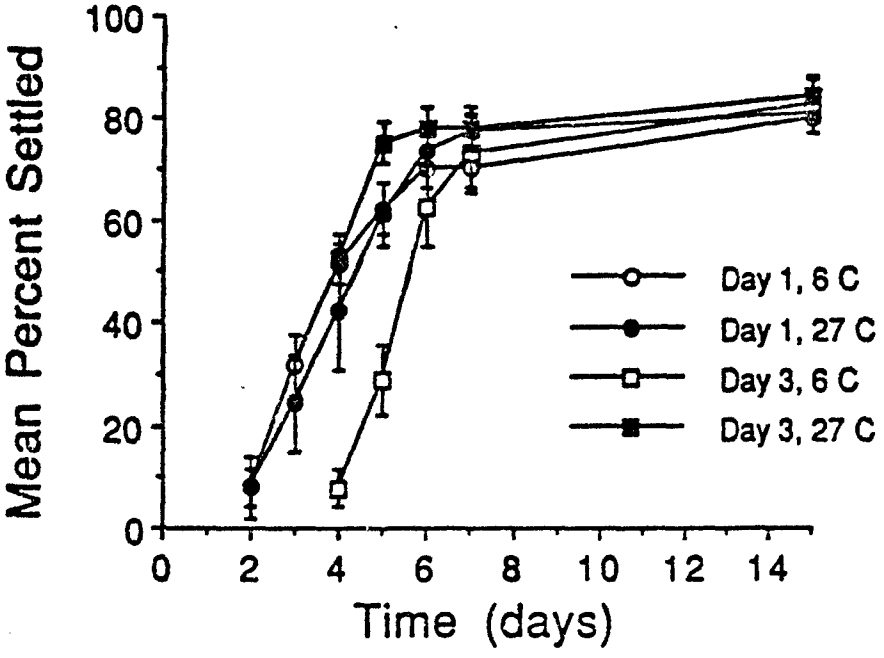
B. eburneus #127 (polystyrene, 20 ppt)



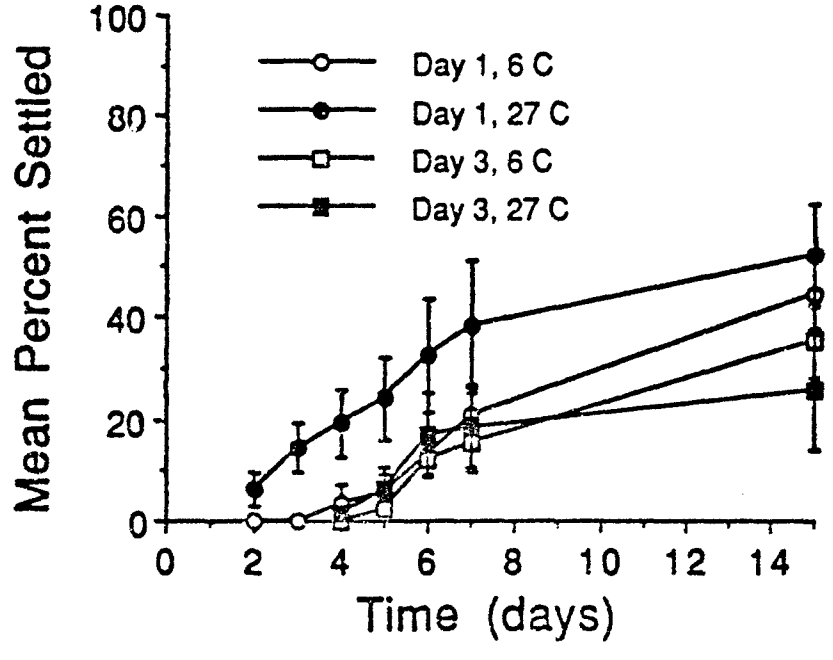
B. eburneus #127 (glass, 20 ppt)



B. improvisus #36 (polystyrene, 15 ppt)



B. improvisus # 36 (glass, 15 ppt)



CONCLUSIONS

- Both *Balanus amphitrite* and *B. improvisus* cyprids settle in consistently high numbers on polystyrene and glass substrata. *B. amphitrite* tends to prefer glass whereas *B. improvisus* prefers polystyrene.
- *B. eburneus* has a variable settlement response and will not act as a good model for settlement assays until assay methods are modified to ensure predictably high settlement.
- These observations of settlement of *B. amphitrite* and *B. improvisus* provide baseline data for experiments in progress on the effects of microbial films on attachment of cyprids to surfaces.

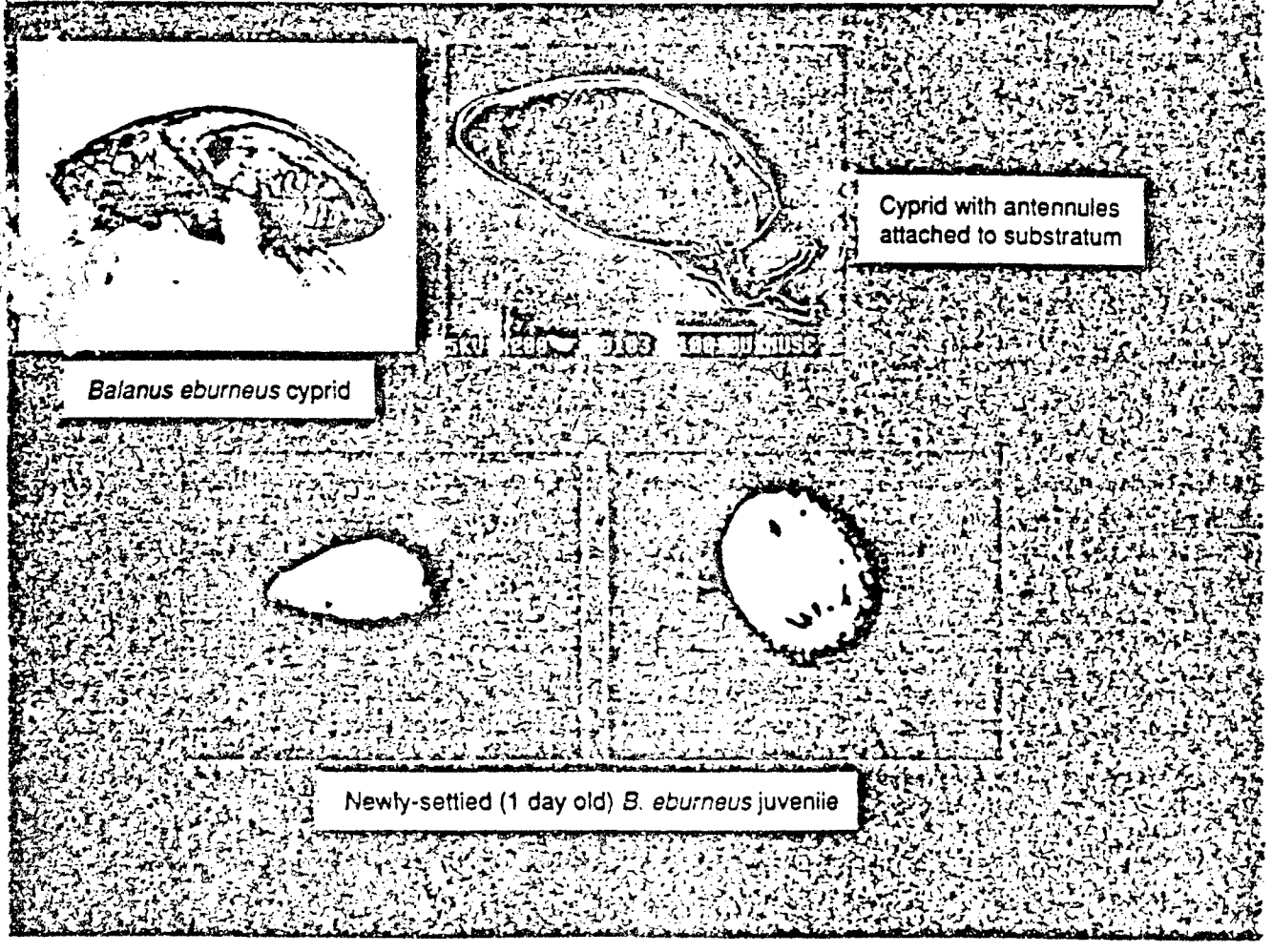
Appendix II

Effects of Surface-Associated Bacteria on Attachment of Barnacle Larvae

Nancy J. O'Connor and Donnia L. Richardson
South Carolina Marine Resources Research Institute

INTRODUCTION

"Slime films" quickly form on substrata submerged in seawater, indicating colonization by microbes. Early colonizers include bacteria, especially gram negative bacilli. Larvae of sessile macroinvertebrates, such as barnacles, searching for substrata on which to settle and metamorphose encounter surfaces covered by bacteria and other microbes. We asked whether bacteria on submerged substrata affect attachment of barnacle larvae.



METHODS

Gram negative bacteria (*Deleya marina*, *Alteromonas macleodii*, and *Pseudomonas fluorescens*) grown in culture to stationary growth phase, or extracellular materials (ECM) present in culture supernatant, were allowed to attach to sterile polystyrene Petri dishes or borosilicate glass vials. Control dishes were incubated with sterile-filtered seawater (FSW). Dishes and vials were rinsed and refilled with FSW and ~25 *Balanus amphitrite* cyprids. Attachment of cyprids to each dish was monitored at 24 (±2) hour intervals for at least 4 days. Assays were repeated three times with different batches of larvae. Data were analyzed using multivariate and repeated measures ANOVA procedures, and differences among treatments at each day of an assay were examined using Tukey's studentized range test.

Key to numbers on graphs showing differences between treatments:

1 = Control > Cells

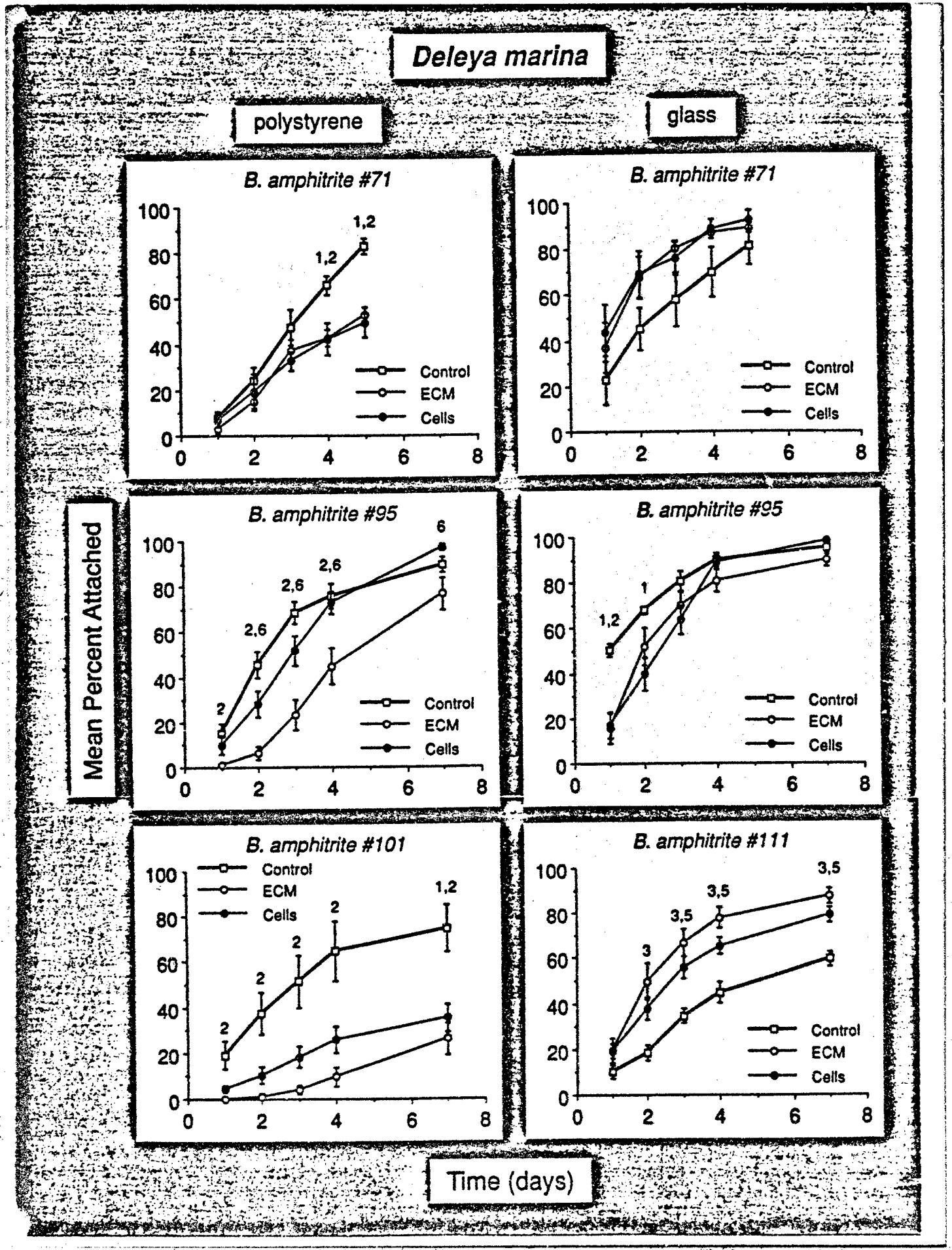
2 = Control > ECM

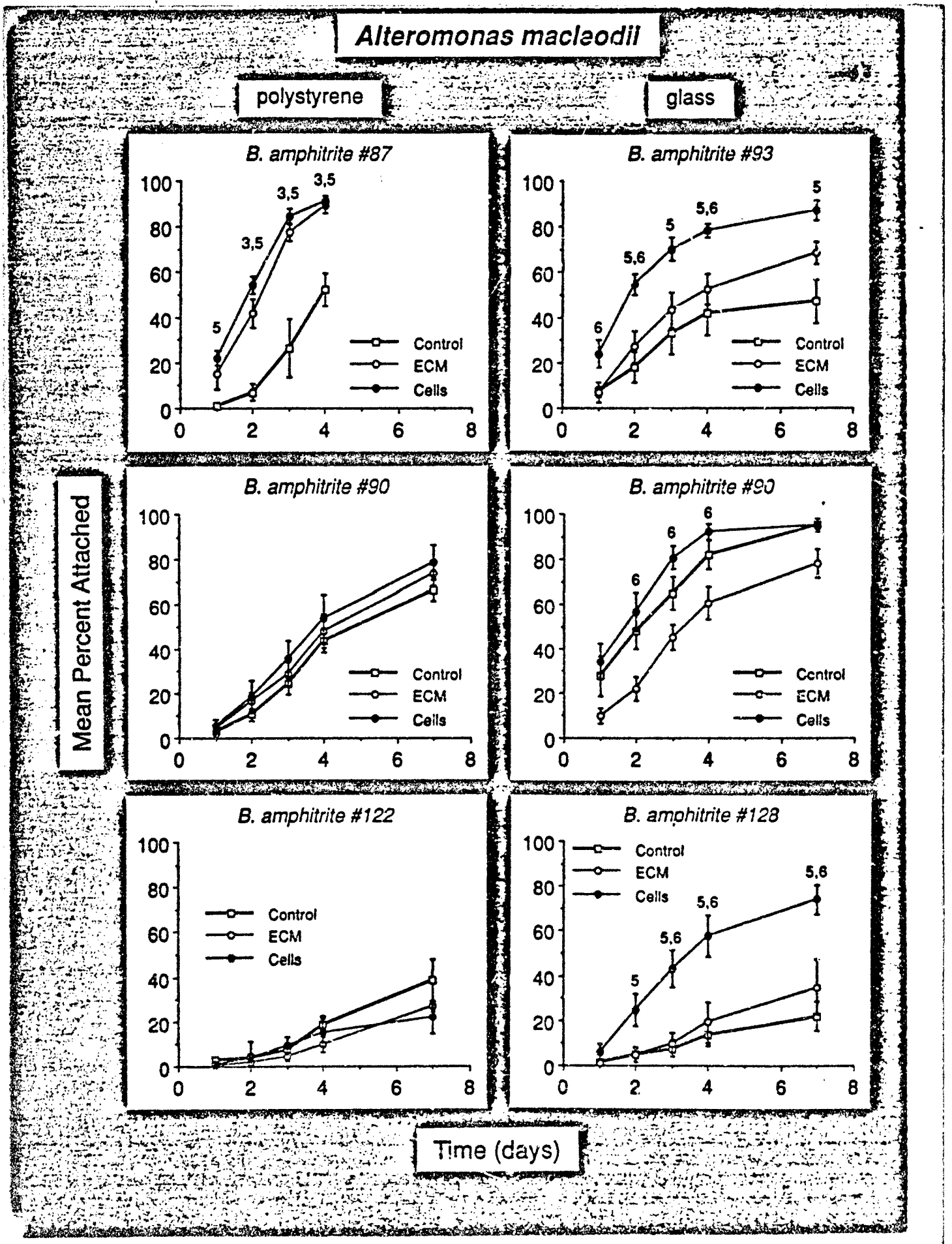
3 = ECM > Control

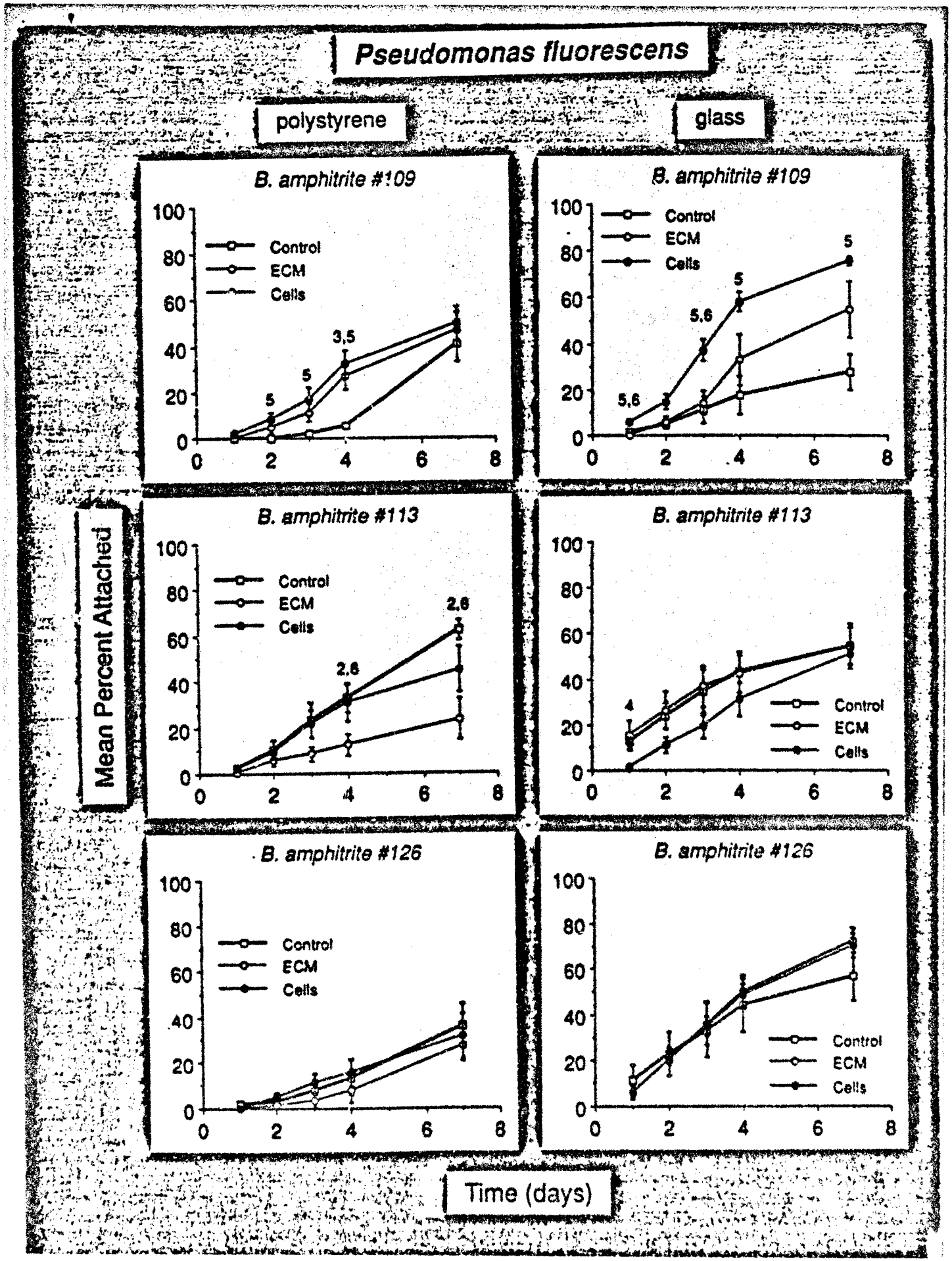
4 = ECM > Cells

5 = Cells > Control

6 = Cells > ECM


Deleya marina
polystyrene
glass
B. amphitrite #71
B. amphitrite #71
B. amphitrite #95
B. amphitrite #95
B. amphitrite #101
B. amphitrite #111
Control
ECM
Cells
Mean Percent Attached
Time (days)


Alteromonas macleodii
polystyrene
glass
B. amphitrite #87
B. amphitrite #93
B. amphitrite #90
B. amphitrite #90
B. amphitrite #122
B. amphitrite #128
Control
ECM
Cells
Mean Percent Attached
Time (days)


Pseudomonas fluorescens
polystyrene
glass
B. amphitrite #109
B. amphitrite #109
B. amphitrite #113
B. amphitrite #113
B. amphitrite #126
B. amphitrite #126
Control
ECM
Cells
Mean Percent Attached
Time (days)

RESULTS AND DISCUSSION

Deleya marina cells and ECM on polystyrene surfaces tended to retard larval attachment in the assay, but mixed results (both stimulation and retardation of attachment) were observed on glass substrata.

Alteromonas macleodii and ECM on polystyrene stimulated attachment in only 1 out of 3 assays. On glass, cells stimulated attachment in 2 out of 3 assays, while ECM had no effect.

Pseudomonas fluorescens cells and ECM had mixed effects on larval attachment. Cells occasionally stimulated attachment on polystyrene and glass, whereas ECM had little effect, retarding attachment in only 1 out of 6 assays.

The high variability and inconsistency of assay results makes interpretation difficult. Variability could be due to several factors, including differences in the chemical signals associated with bacterial of ECM films from one assay to the next. In addition, heterogeneous patterns of attachment of cells and ECM to substrata could explain some of the variability. We plan to examine attachment patterns of cells and ECM this summer.

Assays in progress using *Balanus improvisus* and bacterial cells and ECM indicate no strong differences between its attachment behavior and that of *B. amphitrite*.

Supported by the Office of Naval Research.

Appendix III

PRESENTATIONS AND PUBLICATIONS (Last 12 Months):

1) Poster Presentations-Abstracts:

" A Comparative Study of the Attachment of Marine Bacteria to Polystyrene Surfaces". Dhayalini Chandrasegaram and J. B. Stukes. Forum on Undergraduate Research (1992). Atlanta, GA.

"A Study of the Marine Bacteria Associated with Barnacles In Charleston Harbor". Michelle Haynes, George McCowan, and B.R. Stokes. Forum on Undergraduate Research (1992). Atlanta, GA.

" Purification of Extracellular Polysaccharide from Bacteria Isolated From Charleston Harbor SC". Leyana Lloyd and Frank Weaver. Forum on Undergraduate Research (1992). Atlanta, GA.

"Comparative Larval Settlement Behavior in Three Species of Barnacle (Balanus amphitrite, B. eburneus and B. improvisus)". Crustacean Society Summer Meeting (1992). Charleston, SC. N. O'Connor and D. Richardson.

"Effects of Surface-Associated Bacteria on Attachment of Barnacle Larvae". Benthic Ecology Meeting (1993). Mobile, AL. N. O'Connor and D. Richardson.

2) Slide Presentations-Abstracts

"Adsorption of Marine Bacteria to Polystyrene Surfaces",.Dhayalini Chandrasegaram and J.B. Stukes. SC Academy of Science Meeting (1993). U.S.C., Columbia, SC

"A Study of the Marine Bacteria Associated with Barnacles in Charleston Harbor". Michelle Haynes and B.R. Stokes. S.C. Academy of Science Meeting (1993). U.S.C., Columbia, SC

"Isolation/Characterization of Extracellular Extrudants from Bacteria Taken Charleston Harbor SC". Leyana Lloyd and Frank Weaver. S.C. Academy of Science Meeting (1993). U.S.C., Columbia, SC

3) Invited Presentation:

"Marine Bacteria: Isolation/Characterization and Adhesion to Polystyrene Surfaces". J.Stukes. Benedict College, Columbia, SC Nov. 23, 1992.

4) An article entitled " Minorities Encouraged to Enter Marine Science" describing the ONR program was published in the spring 1993 edition of the newspaper of the SC Wildlife and Marine Resources Department

5) Three issues of the program newsletter, "Synergy", were produced and circulated to HBCUs, interested agencies, and member institutions of the Southern Association of Marine Laboratories.

Appendix IV

STATISTICAL ANALYSES FOR BACTERIAL ATTACHMENT STUDIES

Analysis of Variance of individual bacterial species for time intervals 0.25, 0.5, 1, 2, 24, 72, 96, and 120 hr. (Level of significance is 0.05 at the 95% confidence interval.)

Bacterial Species	Culture Conditions (hr.)	P values
A. macleodii	24	0.047
	48	0.995
D. marina	24	0.258
	48	0.124
P. fluorescens	24	0.120
	48	0.000

Analysis of Variance among *A. macleodii*, *D. marina*, *P. fluorescens* (24 hr. cultured cells) for attachment at the same time interval. (Level of significance is 0.05 at the 95% confidence interval.)

Time Interval (hr.)	P value
0.25	0.003
0.5	0.003
1	0.003
2	0.032
24	0.719
72	0.579
96	0.826
120	0.789

Analysis of Variance among *A. macleodii*, *D. marina*, *P. fluorescens* (48 hr. cultured cells) for attachment at the same time interval. (Level of significance is 0.05 at the 95% confidence interval.)

Time Interval (hr.)	P value
0.25	0.001
0.5	0.002
1	0.001
2	0.008
24	0.000
72	0.079
96	0.157
120	0.172

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